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(54) Title: IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

(57) Abstract: The present invention relates to internalizing peptides which facilitate the uptake and transport of cargo into the cytoplasm and nuclei of cells as well as methods for the identification of such peptides. The internalizing peptides of the present invention are selected for their ability to efficiently internalize cargo into a wide variety of cell types both *in vivo* and *in vitro*. The method for identification of the internalizing peptides of the present invention comprises incubating a target cell with a peptide display library, isolating peptides with internalization characteristics and determining the ability of said peptide to internalize cargo into a cell.

IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND /OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

SPECIFICATION

5 FIELD OF THE INVENTION

The present invention relates to peptides which facilitate the delivery, uptake and transport of proteins, DNA and viruses into the cytoplasm and/or nuclei of cells as well as methods for the identification of such peptides.

BACKGROUND OF INVENTION

The ability to deliver nucleic acids, amino acids, small molecules, viruses, etc. (hereafter referred to collectively as "cargo") to specific cell types is useful for various applications in oncology, developmental biology, gene therapy and in the general understanding of the mode of operation of particular proteins, nucleic acids and small molecules in a model system. There are a number of viral and nonviral delivery systems which have been developed, including vectors derived from human adenoviruses, herpes simplex viruses, adeno-associated viruses, retroviruses (Mulligan, 1993, *Science* 260:926-932; Berns and Giraud, 1995, *Ann. N.Y. Acad. Sci.* 772:95-104; Smith, 1995, *Ann. Rev. Microbiol.* 49:807-838) and others. Nonviral delivery systems include liposomes and conjugates of plasmid and/or DNA with agents designed to facilitate recognition of specific cell surface receptors and protect the newly introduced intracellular DNA from degradation (Wu and Wu, 1987, *J. Biol. Chem* 262:4429-4432; Curiel et al., 1991, *Proc. Natl. Acad. Sci.* 88:8850-8854; Wagner et al., 1992, *Proc. Natl. Acad. Sci.* 89:6099-6103; Zatloukal et al. 1993, *Gene* 135:199-207; Douglas et al., 1996, *Bio/Technology* 14:1574-1578; Zeigler et al., 1996, *Transplantation* 61:812-817; Felgner, 1997, *Sci. Am.* 276:102-106).

The cell recognition specificity of viruses and viral vectors is generally very high, and their ability to transfer genetic material into a target cell makes them

particularly attractive candidates for the delivery of cargo to a target cell. However, there are potential risks and limitations associated with the use of viral vectors for the delivery of cargo, such as the possibility of integration into a host genome by retroviral vectors, and adverse host reactions (*e.g.* immunological reactions) against other viral vectors, such as adenovirus. See, *e.g.*, Yang et al., 1995, *J. Virol.* 69:2004-2015.

Receptor-mediated endocytosis is widely exploited in experimental systems as a natural pathway for the targeted delivery of cargo. Endocytic pathways have been used for selective delivery of therapeutic and other biologically active agents to specific cells and to particular intracellular compartments. See generally, Shen et al., 1992, *Adv. Drug Deliv. Rev.*, 8:93-113; Kato and Sugiyama, 1997, *Crit. Rev. Ther. Drug Carrier Syst.* 14:287-331. In these systems, ligands to cell-specific receptors are either conjugated to cargo, for example, macromolecules (Vitetta et al., 1993, *Immunol. Today* 14:252-259; Kuzel and Rosen, 1994, *Curr. Opin. Oncol.* 6:622-626), liposomes (Kirpotin et al., 1997, *Biochemistry* 36:66-75; Spragg et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:8795-8800), radioisotopes or toxins (Fitzgerald, 1996, *Semin. Cancer Biol.* 7:87-95) and synthetic gene complexes (Wu and Wu, 1993, *Adv. Drug Deliv. Rev.* 12:159-167), or expressed on the surface of viral transfection vehicles (Kozarsky and Wilson, 1993, *Curr. Opin. Genet. Dev.* 3:49-503; Wickham et al., *Gene Ther.* 2:750-756).

Early in the development of receptor-mediated delivery strategies, a ligand was used, together with a polycation (such as polylysine) for the targeting of a condensed DNA to a cell where the ligand was specific for a particular cell surface receptor. See Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432; Wu and Wu, 1988, *J. Biol. Chem.* 263:14621-14624; Wu and Wu, 1989, *J. Biol. Chem.* 264:16985-16987. These strategies suffered from the inability of the DNA to be efficiently released into the cytoplasm, although internalization was successful. The addition of endosomolytic agents, such as adenovirus, improved upon the problems associated with ligand/polycation conjugates, however simplified systems were desired. See generally, Cotton and Wagner in *The Development of Human Gene Therapy* 265 (Cold Spring Harbor Press, Cold Spring Harbor, NY 1999).

The identity of cellular receptors and the mode of their interaction with a ligand-presenting vehicle determine the cell specificity of the delivery system and the intracellular localization of the transported molecules. See Shen et al., 1992, *Adv. Drug Deliv. Rev.* 8:93-113 and Basu, 1990, *Biochem Pharmacol.* 40:1941-1946. This information is useful in the development of simplified methods for delivery.

However, these methods are limited by the ability to transfer sufficient quantities of the molecules to specific cells *in vivo*, although they have proven effective *in vitro*. Sato et al., 1996, *Adv. Drug. Deliv. Rev.* 19:445-467. The application of these methods *in vivo* are limited by several factors, principally the low targeting efficiency of receptor-mediated delivery systems.

Another simplified synthetic system utilized short synthetic peptides based on the sequence thought to be important for membrane fusion by influenza hemagglutinin (Wagner et al., 1992, *Proc. Natl. Acad. Sci.* 89:7934-7938). The inclusion of these peptides into condensed-DNA complexes allowed for improved simplified delivery of the DNA to a cell. However, the limitation of this method was the affinity of the peptide for numerous cell types which also may translate into an inability to transfer sufficient quantities to a specific target cell.

One approach to improving the ability to transfer sufficient quantities of cargo to specific cells is to identify novel cell-targeting ligands, which increase the rate and specificity for the transport of molecules. The first protein discovered having such transduction properties was the HIV transactivator protein, TAT. See Green & Lowenstein, *Cell*, 55:1179-1188 (1988); Frankel & Pabo, *Cell* 55:1189-1193 (1988). Subsequently, an 11 amino acid transduction domain in TAT (TAT-PTD) responsible for the observed transduction properties was identified, based on its high basic residue content. See Fawell et al., *Proc. Natl. Acad. Sci. USA* 91:664-668 (1994). It has been shown that fusion protein constructs containing TAT-PTD are capable of delivering proteins to a wide spectrum of cell types both *in vitro* and *in vivo*. See Nagahara et al., *Nat. Med.* 4:1449-52 (1998); Vives et al., *J. Biol. Chem.* 272:16010-17 (1997); Shwarze et al., *Science* 285:1569-72 (1999); Vocero-Akbani et al., *Nat. Med.* 5:29-33 (1999); Moy et al., *Mol. Biotechnol.* 6:105-13 (1996). It is not known however if TAT-PTD will be effective in all cells and with all fusion constructs. It is possible

Cy3 to be internalized into HIG-82 cells; and (D) shows the ability of peptide 5 linked to M13 phage labeled with Cy3 to be internalized into HIG-82 cells.

Figure 14: shows the ability of the death peptide (SEQ ID NO:24), an antimicrobial apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) and peptide 5 at various concentrations to impair cell viability in HIG 82 Cells.

Figure 15: is a graph showing the ability of the death peptide (SEQ ID NO:24), an antimicrobial apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) and peptide 5 at various concentrations to impair cell viability in HIG 82 Cells as measured by OD₅₇₀ using an MTT assay.

Figure 16 (A) is a graph showing the ability of the death peptide (SEQ ID NO:24) (DP1; ●), antimicrobial peptide (SEQ ID NO:23) (KLA; ■) and peptide 5 (SEQ ID NO:5) (TBS; ▲) to inhibit the growth of MCA205 tumors, (B) shows representative surface morphology of mice with fibrosarcomas when treated with the death peptide (SEQ ID NO:24; DP1) and the antimicrobial peptide (SEQ ID NO:23; KLA) respectively, (C) is hematoxylin and eosin (H&E) staining (left) and TUNEL (right) showing that the death peptide (SEQ ID NO:24; DP1) but not the antimicrobial peptide alone (SEQ ID NO:23; KLA) mediates apoptosis of MCA205 tumors *in vivo*, and (D) is a scatter plot showing individual tumor sizes.

Figure 17 shows the ability of peptide 5 to facilitate the uptake of β -gal into CD34+/LIN- stem cells.

Figure 18 shows TUNEL hematoxylin and eosin staining of tissue from arthritic rabbit knee joints indicating that the death peptide (SEQ ID NO:24; DP1) mediates apoptosis in hyperplastic synovium whereas the antimicrobial peptide alone (SEQ ID NO:23; KLA) does not.

Figure 19 is a bar graph showing that the death peptide (SEQ ID NO:24; DP1) causes great reduction of white blood cells in lavage fluid of IL-1 inflamed rabbit joints.

Figure 20 is a bar graph showing that internalization of p53 into cells via the peptides of the present invention induces p21 promoter driven luciferase expression in a rabbit synovial cell line (Hig-82 cells).

the present invention may be identified by fusion of an internalizing peptide of the present invention with another peptide with a desired function such as, for example, but not by way of limitation, fusion of an internalizing peptide to a previously identified ubiquitin targeting peptide which may have the amino acid sequence
5 GVVGKLGQRRTKKQRRQKK (the "UBI" peptide) as set forth by SEQ ID NO:73 or fusion of an internalizing peptide with an endoplasmaticum reticulum (ER) localization signal such as adenovirus E19 sequence which may have the amino acid sequence GRRTKKQRRQKKPPRYMILGLLALAAVCSAA as set forth by SEQ ID NO:74. In addition, the internalizing peptide of the present invention may have the
10 amino acid sequence GRRTKKQRRQKKPP (SEQ ID NO:75).

Any technique known to those in the art may be employed to identify peptides with high efficiency of internalization for a target cell. In one preferred embodiment of the invention, phage biopanning was employed to select for peptides (Table 1, 2, 3 and 4) which are internalized into such cell lines as Hig-82 cells, human
15 synovial cells, rabbit synovial cells, human primary airway HBE144 cells, primary human islet cells, murine myoblast C2C12 cells, dog kidney epithelial MDCK cells, murine tumor MCA 205 cells, murine tumor MC38 cells (all shown in Figure 6) and rabbit synovial lining (Figure 4). In addition, the internalizing peptides are internalized into mucosa, such as cervical mucosa (*see* Figure 22, and Example 2 and
20 9 below). In another preferred embodiment, the internalizing peptides of the present invention can be fused to another peptide with a desired function (*e.g.* ubiquitin targeting or ER localization functions) such as the internalizing peptides set forth by SEQ ID NOs:73 and 74 which are also internalizing into a wide variety of cells including, but not limited to Hig-82 cells, human synovial cells, rabbit synovial cells,
25 human primary airway HBE144 cells, primary human islet cells, murine myoblast C2C12 cells, dog kidney epithelial MDCK cells, murine tumor MCA 205 cells, murine tumor MC38 cells, rabbit synovial lining, and mucosa, such as cervical mucosa (*see* Figure 22, and Example 2 and 9 below).

TABLE 1

	1)	peptide 1 (pep1)	KRIQRILSRNS	(SEQ ID NO:1)
	2)	peptide 2 (pep2)	KRIHPRLTRSIR	(SEQ ID NO:2)
	3)	peptide 3 (pep3)	PPRLRKRRQLNM	(SEQ ID NO:3)
5	4)	peptide 4 (pep4)	PIRRRKKLRLK	(SEQ ID NO:4)
	5)	peptide 5 (pep5)	RRQRRTSKLMKR	(SEQ ID NO:5)
	6)	peptide 6 (pep6)	MHKRPTTPSRKM	(SEQ ID NO:6)
	7)	peptide 7 (pep7)	RQRSRRRPLNIR	(SEQ ID NO:7)
	8)	peptide 8 (pep8)	RIRMIQNLIKKT	(SEQ ID NO:8)
10	9)	peptide 9 (pep9)	SRRKRQRSNMRI	(SEQ ID NO:9)
	10)	peptide 10 (pep10)	QRIRKSKISRTL	(SEQ ID NO:10)
	11)	peptide 11 (pep11)	PSKRLHNNLRR	(SEQ ID NO:11)
	12)	peptide 12 (pep12)	HRHIRRQSLIML	(SEQ ID NO:12)
	13)	peptide 13 (pep13)	PQNRLQIRRHKS	(SEQ ID NO:13)
15	14)	peptide 14 (pep14)	PPHNRIQRRLLNM	(SEQ ID NO:14)
	15)	peptide 15 (pep15)	SMLKRNHSTSNR	(SEQ ID NO:15)
	16)	peptide 16 (pep16)	GSRHPSLIIPRQ	(SEQ ID NO:16)
	17)	peptide 17 (pep17)	SPMQKTMNLPPM	(SEQ ID NO:17)
	18)	peptide 18 (pep18)	NKRILIRIMTRP	(SEQ ID NO:18)
20	19)	peptide 19 (pep19)	HGWZIHGLLHRA	(SEQ ID NO:25)
	20)	peptide 20 (pep20)	AVPAKKRZKSV	(SEQ ID NO:26)
	21)	peptide 21 (pep21)	PNTRVRPDVSF	(SEQ ID NO:27)
	22)	peptide 22 (pep22)	LTRNYEAWVPTP	(SEQ ID NO:28)
	23)	peptide 23 (pep23)	SAETVESCLAKSH	(SEQ ID NO:29)
25	24)	peptide 24 (pep24)	YSHIATLPFTPT	(SEQ ID NO:30)
	25)	peptide 25 (pep25)	SYIQRTPSTTLP	(SEQ ID NO:31)
	26)	peptide 26 (pep26)	AVPAENALNNPF	(SEQ ID NO:32)
	27)	peptide 27 (pep27)	SFHQFARATLAS	(SEQ ID NO:33)
	28)	peptide 28 (pep28)	QSPTDFTFPNPL	(SEQ ID NO:34)
30	29)	peptide 29 (pep29)	HFAAWGGWSLVH	(SEQ ID NO:35)
	30)	peptide 30 (pep30)	HIQLSPFSQSWR	(SEQ ID NO:36)

31) peptide 31 (pep31) LTMPSDLQPVLW (SEQ ID NO:37)

TABLE 2

	1)	peptide 32 (pep32)	FQPYDHPAEVSY	(SEQ ID NO:38)
	2)	peptide 33 (pep33)	FDPFFWKYSPRD	(SEQ ID NO:39)
5	3)	peptide 34 (pep34)	FAPWDTASFMLG	(SEQ ID NO:40)
	4)	peptide 35 (pep35)	FTYKNFFWLPEL	(SEQ ID NO:41)
	5)	peptide 36 (pep36)	SATGAPWKMWVR	(SEQ ID NO:42)
	6)	peptide 37 (pep37)	SLGWMLPFSPPF	(SEQ ID NO:43)
	7)	peptide 38 (pep38)	SHAFTWPTYLQL	(SEQ ID NO:44)
10	8)	peptide 39 (pep39)	SHNWLPLWPLRP	(SEQ ID NO:45)
	9)	peptide 40 (pep40)	SWLPYPWHVPSS	(SEQ ID NO:46)
	10)	peptide 41 (pep41)	SWWTPWHVHSES	(SEQ ID NO:47)
	11)	peptide 42 (pep42)	SWAQHLSLPPVL	(SEQ ID NO:48)
	12)	peptide 43 (pep43)	SSSIFPPWLSFF	(SEQ ID NO:49)
15	13)	peptide 44 (pep44)	LNVPWSWFLSQR	(SEQ ID NO:50)
	14)	peptide 45 (pep45)	LDITPFLSLTLP	(SEQ ID NO:51)
	15)	peptide 46 (pep46)	LPHPVLHMGPLR	(SEQ ID NO:52)
	16)	peptide 47 (pep47)	VSKQPYMWNGN	(SEQ ID NO:53)

TABLE 3

20	1)	peptide 48 (pep48)	NYTTYKSHFQDR	(SEQ ID NO:54)
	2)	peptide 49 (pep49)	AIPNNQLGFPEK	(SEQ ID NO:55)
	3)	peptide 50 (pep50)	NIENSTLATPLS	(SEQ ID NO:56)
	4)	peptide 51 (pep51)	YPYDANHTRSPT	(SEQ ID NO:57)
	5)	peptide 52 (pep52)	DPATNPGPHFPR	(SEQ ID NO:58)
25	6)	peptide 53 (pep53)	TLPSPLALLTVH	(SEQ ID NO:59)
	7)	peptide 54 (pep54)	HPGSPFPPEHRP	(SEQ ID NO:60)
	8)	peptide 55 (pep55)	TSHTDAPPARSP	(SEQ ID NO:61)
	9)	peptide 56 (pep56)	MTPSSLSTLPWP	(SEQ ID NO:62)
	10)	peptide 57 (pep57)	VLGQSGYLMMPMR	(SEQ ID NO:63)

TABLE 4

	1)	peptide 58 (pep58)	QPIIITSPYLPS	(SEQ ID NO:64)
	2)	peptide 59 (pep59)	TPKTMQTQTYDFS	(SEQ ID NO:65)
	3)	peptide 60 (pep60)	NSGTMQSASRAT	(SEQ ID NO:66)
5	4)	peptide 61 (pep61)	QAASRVENYMHHR	(SEQ ID NO:67)
	5)	peptide 62 (pep62)	HQHKPPPLTNNW	(SEQ ID NO:68)
	6)	peptide 63 (pep63)	SNPWDSL SVST	(SEQ ID NO:69)
	7)	peptide 64 (pep64)	KTIEAHPPYYAS	(SEQ ID NO:70)
	8)	peptide 65 (pep65)	EPDNWSLDFPRR	(SEQ ID NO:71)
10	9)	peptide 66 (pep66)	HQHKPPPLTNNW	(SEQ ID NO:72)

Of the peptides of Table 4, three have homology to known proteins.

Pep63 (SEQ ID NO:69) is homologous to a bacterial protein methenyl tetrahydromethanopterin cyclohydrolase of xanthobacter autotrophicus (Genbank
 15 Accession Number AF139593). Pep65 (SEQ ID NO:71) is homologous to a yeast
 hypothetical protein in the MPP10-SAG1 intergenic region of Saccharomyces
 cerevisiae (Genbank Accession Number NP012536.1). Additionally, pep66 (SEQ ID
 NO:72) is homologous to herpesvirus 1 probably nuclear antigen protein (Genbank
 Accession Number P33485).

20 Additionally, any nucleotide sequences which encode the peptides
 represented by SEQ ID NOs:1-18 and 25-72 of the present invention are also
 contemplated by the present invention.

The internalizing peptides of the present invention are cationic
 (positively charged) as are TAT-PTD and antennapedia peptide (Ant-PTD). Table 5
 25 below indicates the positive nature of pep 1-6 (SEQ ID NO:1-6), TAT-PTD (SEQ ID
 NO:21), Ant-PTD (SEQ ID NO:19) and a random control peptide (SEQ ID NO:20).
 Peptides 1-6 are all positively charged and enriched for lysine and arginine residues
 (Table 5 below).

TABLE 5

	<u>Peptide</u>	<u>SEQ ID NO.</u>	<u>Length</u>	<u>MW</u>	<u>Lys + Arg/L</u>
5	Pep 1	SEQ ID NO:1	12	1482.81	0.333
	Pep 2	SEQ ID NO:2	12	1531.88	0.417
	Pep 3	SEQ ID NO:3	12	1563.94	0.417
	Pep 4	SEQ ID NO:4	12	1619.81	0.667
	Pep 5	SEQ ID NO:5	12	1614.98	0.583
	Pep 6	SEQ ID NO:6	12	1468.80	0.333
10	Random	SEQ ID NO:20	12	1280.40	0.167
	TAT-PTD	SEQ ID NO:21	11	1558.63	0.727
	Ant-PTD	SEQ ID NO:19	16	2245.78	0.438

Where desired, the internalizing peptides of the present invention may also facilitate the nuclear translocation of cargo.

15 The usefulness of the present invention may be demonstrated, for example, by incubating a complex comprising an internalizing peptide linked to cargo with target cells and measuring the efficiency of transfer of the peptide-cargo complex to the target cell. In one embodiment, the selected internalizing peptide was biotinylated and coupled to streptavidin-labeled β -galactosidase (the "cargo"). The
20 ability of the internalizing peptide to internalize β -gal into a cell was established by adding X-gal to cells, which when in the presence of β -gal is cleaved and gives a blue color. Cells which stained blue indicated that β -gal had successfully been transferred to the cells via the peptides of the present invention (Fig. 1-6 and 12) (see Example 4 below). In addition, a polynucleotide encoding one of the peptides (peptide 5; SEQ
25 ID NO:5) was subcloned with nucleic acid encoding eGFP (green fluorescent protein) to produce a peptide-eGFP fusion, expressed and purified from bacteria. Peptide 5, when fused to eGFP, facilitated the internalization of eGFP into cells (see Figure 9), which was directly monitored by fluorescence microscopy.

In a preferred embodiment of the invention, the internalizing peptides
30 which allow for the co-entry of peptide-linked cargo, and the translocation of the

cargo to the nuclei are pep 2 (SEQ ID NO:2), pep 3 (SEQ ID NO:3), pep 4 (SEQ ID NO:4), pep5 (SEQ ID NO:5) shown in Table 1 above, and UBI (SEQ ID NO:73).

The internalizing peptides of the present invention may be linked to cargo by any method known to those in the art, such as, but not limited to chemical cross-linking, avidin bridge, glutathione-S-transferase bridge, peptide-cargo fusion protein, etc. The peptides of the present invention may also be synthesized as a fusion with a peptide nucleic acid (PNA) which is a DNA mimic capable of forming double and triple helices with DNA (see Knudsen and Nielsen, 1997, *Anticancer Drugs* 8:113-118). This peptide-PNA fusion can form a stable DNA or RNA/PNA duplex (Branden et al., 1999, *Nat. Biotechnol.* 17:784-787) which may enter cells via the peptides of the present invention, thereby delivering DNA or RNA to a target cell.

Additionally, the ability of the internalizing peptide to carry the cargo into the cell may be measured by the presence of functional cargo in the cell (e.g. the presence of β -gal may be demonstrated by the ability of the cell to cleave X-gal and give a blue color; the presence of cystic fibrosis transmembrane regulator (CFTR) protein may be demonstrated by the presence of a functional chloride ion channel in a cell originally lacking CFTR, and the presence of an apoptotic factor may be shown by the apoptosis of cells after the administration of a peptide-apoptosis factor construct of the present invention). The cargo (e.g. polypeptide, polynucleotide, small molecule, virus, plasmid) may be labeled by a method known in the art (e.g. radiolabeling or fluorescent labeling) and the presence of the label would establish the efficient delivery of the cargo into the target cell by the internalizing peptide. In addition, the presence of an immunogen in the cell of a subject may be measured by the ability to elicit an immune response in a subject.

To establish nuclear translocation of the internalizing peptides themselves and the ability of the internalizing peptide to transfer a small molecule linked thereto to a cell, the peptides were labeled with streptavidin-Cy3, a fluorescent marker (see Example 4 below). Using confocal microscopy, the ability of the peptide to translocate to the nucleus is determined. Other methods known in the art of establishing the presence of a peptide in the cytoplasm or nucleus of a cell are also contemplated by the present invention (e.g. labeling of the peptide with a

radioisotope, a fluorescent marker or a dye).

The internalizing peptides of the present invention facilitate uptake and delivery into a wide variety of cell types (see Figures 1-4 and 6) including cells which are refractory to virus infection, such as primary human airway epithelial cells (Figure 6), as well as other types of primary and established cell lines, such as Hig-82 cells (a rabbit synovial cell line established by Christopher Evans, University of Pittsburgh, ATCC Deposit No. CRL-1832), rabbit synovial cells, human synovial cells, primary human islet cells, murine myoblast cells, dog kidney epithelial cells, murine fibroblast cells, and murine tumor cells (Figures 1-4 and 6) (see Examples 4 and 5 below), cells of different germinal layers, as well as mucosa, such as cervical mucosa (Figure 22).

The peptides of the present invention are also useful for delivery of cargo into cells *in vivo* and can facilitate *in situ* or localized delivery of cargo *in vivo* (see figure 4 and Example 3 below). In one embodiment, a biotinylated peptide-streptavidin- β -gal complex was injected into synovial lining (knee joint) of rabbits, which was then harvested. The harvested synovial lining was then incubated with X-gal to show that the peptide facilitated the uptake of β -gal by synovial lining cells *in vivo*. Ghivizzani et al. (*J. Immunol.* 159:3604 (1997)) have described using the synovial lining of rabbits as a model system for studying arthritis (see also Nita et al., *Arthritis Rheum* 39:820 (1996); Ghivizzani et al., *Proc. Natl. Acad. Sci. USA* 95:4613 (1998); and Ghivizzani et al., *Gene Ther.* 4:977 (1997)). Rheumatoid arthritis is correlated with an excessive proliferation of synovial cells and an apparent defect in synovial cell death that would ordinarily reduce the synovial cell number. Because the peptides of the present invention can facilitate the uptake of cargo into synovial lining cells *in vivo*, the peptides are useful in the alleviation of arthritis. One approach to alleviating rheumatoid arthritis in a subject is to induce synovial cell death. See Wakisaka et al., *Clin. Exp. Immunol.* 114:119-128 (1998); Sakai et al., *Arthritis Rheum.* 41:1251-1257 (1998).

The peptides of the present invention, as well as TAT-PTD, can induce apoptosis in rheumatoid arthritis synovial cells when linked to an apoptosis factor. For example, the peptides of the present invention, as well as TAT-PTD, when linked to an apoptosis factor (e.g. p53; caspase-3; an antimicrobial peptide such as

KLAKLAK (SEQ ID NO:22) and KLAKLAKKLAKLAK (SEQ ID NO:23), which disrupts the mitochondrial membrane once inside a cell (*see* Ellerby et al., *Nat. Med.* 5:1032 (1999); are useful for delivering the apoptosis factor, or a DNA encoding an apoptosis factor, to arthritic joints and inducing apoptosis therein (*see* Figure 18 and Example 7 below). In addition, the peptides of the present invention, as well as TAT-PTD, are useful for delivering apoptosis factors to tumor cells and inducing apoptosis therein. The induction of apoptosis in tumor cells is useful for the destruction of the tumor cell and for increasing the efficacy of drugs designed to treat cancer which are ineffective in tumor cells resistant to apoptosis. *See* Brown and Wouters, *Cancer Res.* 59:1391-1399 (1999); Yamabe et al., *Gene Ther.* 6:1952-1959 (1999). When the antimicrobial peptide, KLAKLAKKLAKLAK (SEQ ID NO:23), is coupled to the peptides of the present invention or TAT-PTD (e.g. the "death peptide" = peptide 5, SEQ ID NO:5, a linker and KLAKLAKKLAKLAK (SEQ ID NO:23) resulting in RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24)) and administered to HIG 82 cells, apoptosis was induced in the cells (*see* Figures 14 and 15 and Example 7 below). Furthermore, when the "death peptide" was intra-tumorally injected subcutaneously into day 7-14 MCA205 (a murine fibrosarcoma cell line) established tumors in mice (the following references describe using MCA205 cells to establish tumors in mice for a model system for studying cancer: Hiroishi et al., *Gene Ther.* 6:1988 (1999); Osaki et al., *Gene Ther.* 6:808 (1999); Nishioka et al., *Cancer Res.* 59:4035 (1999); Gambotto et al., *Cancer Gene Ther.* 6:45 (1999); Kim et al., *Cancer Immunol Immunother.* 47:257 (1999); Mangency and Heidmann, *Proc. Natl. Acad. Sci. USA* 95:14920 (1998); Noffz et al., *J. Immunol.* 160:345 (1998); Osaki et al., *J. Immunol.* 160:1742 (1998); Cayeux et al., *J. Immunol.* 158:2834 (1997); Ohno et al., *J. Immunol.* 156:3875 (1996)), there was shrinkage of the tumor with significant apoptosis/necrosis, especially in the middle of the tumor (*see* Figure 16A, 16B, 16C and Example 7 below).

As noted above, the internalizing peptides of the present invention are useful for delivering and internalizing other apoptotic factors as well, including p53. When p53 was fused to pep5 (SEQ ID NO:5), the p53 was effectively internalized into a rabbit synovial cell line (Hig-82) and able to induce p21 promoter driven

luciferase expression from a reporter plasmid therein (*see* Figure 20 and Example 7 below). The pep5-p53 complex was similar in its ability to induce reporter plasmid expression as a plasmid which expresses p53 and much more effective than an adenovirus vector expressing p53 (*see* Figure 20 and Example 7 below). Due to its apoptotic abilities, the internalizing peptide-p53 complex of the present invention is useful in the treatment of cancer and arthritis and may be administered to a subject, for example, by either local or systemic injection (such as intra-tumoral injection or intra-articular injection).

The "death peptide" is also useful for the induction of apoptosis in other cells, including synovial lining cells. When the death peptide was injected in the arthritic rabbit knees, it mediated apoptosis of the hyperplastic synovium (*see* Figure 18 and Example 7 below). Internalization of apoptosis factors using the peptides of the present invention and TAT-PTD is advantageous since cellular uptake of cargo mediated by the peptides of the present invention is more efficient than viral vector mediated gene transfer or the commercially available antennapedia peptide (Example 3 and Figure 4A).

Rheumatoid arthritis (RA) is a chronic inflammatory disease which is characterized by hyperplasia of the synovial lining of cells, angiogenesis, and infiltration of mononuclear cells resulting in pannus formation, cartilage erosion and ultimately joint destruction. Most of articular cartilage consists of collagens and proteoglycans whose degradation is initiated extra- or peri- cellularly by proteinases produced locally by cells in a around the joint. *See Evans, Agents Actions Suppl.* 32:135-152 (1991). Proteinases, and particularly serine proteinases and neutral mettaloproteinases, are involved in the degradation of articular cartilage. Mesenchymal cells of the joint and white blood cells which colonize the joint during the inflammatory response synthesize various proteinases which degrade articular cartilage. Therefore, reduction of white blood cells at the site of inflammation in arthritic joints is an approach to anti-erosive therapy in arthritis. The internalizing peptides of the present invention are useful in delivering apoptotic factors to cells in arthritic joints, including white blood cells in lavage fluid of inflamed arthritic joints. Figure 19 shows that injection of the death peptide (SEQ ID NO:24; DP1) into

inflamed rabbit joints causes a great reduction of the number of white blood cells in the lavage fluid of IL-1 inflamed rabbit joints (*see also* Example 7 below). White blood cell reduction is useful to reduce swelling, synovial proliferation and cartilage degradation in arthritic joints.

5 Delivery of apoptosis factors via the peptides of the present invention is rapid and potent. For example, low concentrations of the death peptide (SEQ ID NO:24) are required to mediate cell death. In one embodiment of the invention, cell death may be mediated by the internalizing peptides of the present invention (SEQ ID NOs:1-18 and 25-72) linked to cargo comprising an apoptosis factor wherein the concentration administered to cells is between 1 μ M and 1mM. In a preferred embodiment of the present invention, the concentration of the peptide + cargo administered to cells is between 10 μ M and 100 μ M.

In another aspect of the invention, the immune response against tumors may be augmented by co-administration of the internalizing peptides of the present invention linked to a cargo (*e.g.* apoptosis factor) with cytokines and other activating molecules (*e.g.* Flt-3). The cytokines and other activating molecules may be administered to cells via the peptides of the present invention or by any other conventional means of administration known to those of skill in the art.

Additional potential applications for the peptides of the present invention when linked to cargo comprising an apoptosis factor may include the treatment of accessible head and neck tumors, papillomas and other solid tumors, or as an adjuvant therapy in conjunction with radiotherapy, standard chemotherapy or surgical debulking to extend excision margins.

The peptides of the present invention are also useful for developing improved immunogens. For example, the peptides of the present invention may facilitate delivery of, *inter alia*, proteins, polypeptides, DNA, RNA, vectors, and viruses to target cells in a subject which may be useful as immunogens. The peptide/cargo complexes of the present invention are capable of eliciting an immune response when administered to a target cell of a subject. In one embodiment of the invention, the immunogens are vaccines.

While intense efforts have been made in engineering vaccines for HIV in the past decade, an effective vaccine has yet to be developed. The peptides of the present invention may be useful for the development of an effective vaccine for HIV. It is one object of the present invention to provide a vaccine for HIV which is
5 effective at mucosal portals of entry and is capable of eliciting an immune response when delivered to target cells.

The existence of a "common mucosal immune compartment" distinct from systemic immunity is well documented. See Miller et al., *Lab. Invest.* 68:129-145 (1993) and James, *New Generation Vaccines*, edited by Levine M., Woodrow
10 GC, Kaper JB and Cobon BS. Marcel Dekker, Inc., New York pages 151-171 (1995). The mucosal immune system is compartmentalized into "inductive" sites of mucosally associated lymphoid tissue (e.g. Peyer's Patches) where antigen priming occurs and "effector" sites (e.g. lamina propria and epithelium of mucosal tissue) where primed mature effector cells protect against invasion of foreign agents. See Haneberg
15 et al., *Adv. Exp. Med. Biol.* 371A:107-109 (1995). In the intestine, antigen-stimulated induction of naive T and B lymphocytes in the Peyer's patches is followed by trafficking of these cells through adjacent draining lymph nodes (e.g. mesenteric lymph nodes). Fully mature effectors finally traffic to remote lamina propria of mucosal tissues via circulation through the thoracic duct and blood.

20 The present invention provides a method of eliciting an immune response and for immunogens (such as HIV vaccines). Since the primary mode of transmission of HIV is via sexual intercourse, the immunogens of the present invention can induce specific mucosal immune responses.

The immunogens of the present invention preferably comprise an
25 internalizing peptide portion linked to cargo (e.g. antigen). The immunogens of the present invention can present antigen directly to any target cell (e.g. mucosal inductive sites).

The immunogens of the present invention can efficiently induce an immune response, e.g. T helper cell type 1 (TH1) immune responses. T-cells
30 recognize antigens only if they are presented in the form of short, linear peptides (epitopes) in the cleft of major histocompatibility complex (MHC) molecules on the

cell surface. Therefore, if antigens are to be recognized, they must first be processed into short, linear peptides. Most proteins in the cytosol are cleaved by proteases within proteosome complexes into short peptides and carried by transporter proteins into the endoplasmic reticulum (ER). In the ER, the peptides are bound to MHC molecules that are synthesized in the ER. The MHC molecules are then transported to the cell surface where the peptide bound to the MHC molecule is recognized by T-cell receptors (*see* Figure 23).

Figure 23 demonstrates that transduction of a target cell, *e.g.*, dendritic cells, with the peptide/cargo complex of the present invention can promote processing and subsequent presentation of epitopes on the surface of the target cell. To demonstrate that the peptide/cargo complex of the present invention can promote processing and subsequent presentation of epitopes on the surface of a target cell, UBI (SEQ ID NO:73) was fused to a peptide comprising Gp100₂₀₉₋₂₁₇ HLA-A2-restricted epitope, HIV p17₂₃₋₂₁ HLA-A2-restricted epitope and chicken ovalbumin (OVA) epitope (termed 3-Epi) which was in turn fused to green fluorescent protein (eGFP), to make UBI-3Epi-eGFP (*see* Figure 24). As seen in Figure 23, the UBI-3Epi-eGFP fusion was efficiently degraded by the proteasome complex in dendritic cells since the degradation is blocked by the proteasome inhibitor MG132 (*see* Figure 23A). Furthermore, class I presentation and T cell specific recognition of epitope on the surface of mouse dendritic cells is shown in Figure 23B using D16.25 antibody staining which recognizes the OVA epitope of UBI-3Epi-eGFP.

CD8+ cytotoxic T-lymphocytes generally recognize 8-11mer peptides on MHC class I molecules, whereas CD4+ T-helper cells generally recognize 15-25mer peptides on MHC class II molecules. The presentation of the short, linear peptides of the antigen on the cell surface by MHC molecules allows for the initial steps required for T-cell activation. Once T-cell activation is achieved, a potent immune response may be elicited.

The present invention provides for immunogens which may comprise an internalizing peptide of the present invention linked to cargo such as a protein representing an antigen or a DNA or RNA encoding for an antigen. The present invention also provides for a method for eliciting an immune response to a target cell,

said method comprising delivering an immunogen comprising an internalizing peptide and a cargo (preferably an antigen) to a target cell. Once the immunogen is delivered to the target cell, the cargo may be processed (e.g. where the cargo is an antigen, the cargo is proteolyzed into short, linear peptides, or, where the cargo is an RNA or
5 DNA encoding an antigen, the cargo is expressed and then proteolyzed into short, linear peptides) and presented to the cell surface thereby eliciting an immune response (see Figure 23). The target cell may be a mucosal cell such as a cervical mucosal cell (see Figure 22)

In a preferred embodiment of the invention, the immunogen comprises
10 pep5 (SEQ ID NO:5) linked to cargo, such as an antigen. In another preferred embodiment of the invention, the immunogen comprises the UBI peptide (SEQ ID NO:73) linked to cargo, such as an antigen. In a further preferred embodiment of the invention, the immunogen comprises SEQ ID NO:74 linked to cargo, such as an antigen.

15 The cargo portion of the immunogens of the present invention may be an antigen capable of eliciting an immune response to HIV exposure, such as, *inter alia*, an HIV envelope protein, HIV Gag, HIV Pol, HIV Env, HIV Tat, HIV Nef, HIV Vpr, HIV Vpv and HIV Rev. Or the cargo portion of the immunogens of the present invention may be any antigen capable of eliciting a desired immune response.

20 The immunogens of the present invention and the methods of the present invention for eliciting an immune response in a subject can also be accomplished by *ex vivo* transduction of target cells followed by the presentation of the transduced cells to a subject by, for example, intra-muscular or intra-dermal injection or any other technique known to the skilled artisan.

25 The method of the present invention for eliciting an immune response in a subject comprises administering to a target cell of said subject (whether *in vitro*, *in vivo*, or *ex vivo*) a peptide/cargo complex of the present invention wherein said peptide is selected from SEQ ID NOs:1-74 and the cargo is an antigen.

The peptide-cargo complexes of the present invention may be
30 administered to a wide variety of cell types *in vivo*, *in vitro*, and *ex vivo* including, *inter alia*, epithelial cells, tumor cells, hepatocytes, endothelial cells, neurons, muscle,

T-cells, dendritic cells, β cells, primary cells, differentiated cells, stem cells, antigen presenting cells, mucosa, etc by methods known to those skilled in the art.

When administered to stem cells (*e.g.* hematopoietic, muscle, brain, etc.), the peptide-cargo complexes of the present invention can induce differentiation of the stem cells. The peptide cargo complex comprises factors which can stimulate differentiation of stem cells, such as the transcription factor MyoD. Stem cells isolated from bone marrow have been shown to differentiate into a wide variety of tissues, including cartilage and bone, and may be useful therapeutically. See Pittenger et al., *Science* 284:143 (1999).

In addition, the peptide-cargo complex may be used to expand a stem cell population. The internalizing peptides of the present invention can deliver proteins to CD34+ hematopoietic progenitor stem cells (*see* Figure 17 and Example 4). The delivery of immortalizing proteins, such as SV40 T-antigen, HPV E6, HPV E7 and telomerase, can facilitate the transient expansion of stem cell populations. Since the delivery of the immortalizing proteins using the peptides of the present invention is transient and reversible (*e.g.* delivery of the immortalizing protein which will be degraded subsequently in the cell), such delivery offers an advantage in that the stem cell status may be maintained (*i.e.* the cells may be transiently immortalized) while increasing the number of cell doublings that may be achieved. Stable delivery of immortalizing factors may also be achieved by the delivery of cargo encoding the immortalizing factor, *e.g.* a viral vector, plasmid, DNA. This approach can be used to expand a wide variety of stem cells in culture for transplant applications since the peptides of the present invention can facilitate the uptake and delivery of cargo linked thereto to a variety of cells (*see* Example 3 and Figure 6).

Similarly, the peptides of the present invention may be used for expanding differentiated cells (*e.g.* β cells in pancreatic islets, neurons, chondrocytes, etc) which also have a finite number of cell doublings in culture. The peptides of the present invention enter and facilitate the internalization of cargo in differentiated cells, such as islet β cells, (*see* Example 3, and Figure 6) without affecting the ability of the islet cells to respond to signals which are indicative of differentiated function, such as glucose. The proliferation of differentiated cells may be induced by

delivering immortalizing factors (*e.g.* SV40 T-antigen, HPV E6, HPV E7 and telomerase), and particularly SV40 T-antigen, complexed to the peptides of the present invention. The delivery may be transient (delivery of the protein) or may be stable (delivery of a DNA, viral vector, or plasmid encoding the immortalization factor).

It is also an object of the present invention to provide a construct comprising a peptide of the present invention linked to an antigen which can be taken up efficiently by a number of antigen presenting cells (*e.g.* dendritic cells) both *in vivo* and *in vitro* and stimulate an immune response. The peptides may be linked to, *inter alia*, viral antigens (*e.g.*, HIV antigens such as Gag, Pol, Env; HPV-E6; HPV-E7; EBV-LMP1; EBV-LMP2; EBNA1; EBNA3A; EBNA3C; etc), ovalbumin, differentiation antigens (*e.g.*, MART-1/Melan A, gp100, tyrosinase, TRP-1, TRP-2, etc.), tumor specific multilineage antigens (*e.g.*, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15, etc), antigens expressed uniquely by an individual's tumor (*e.g.*, mutated gene products such as p53, CDK4, p16, p21, etc.). In one embodiment, the peptides of the present invention are linked to SIV antigens and are delivered *in vivo* to monkeys to determine the efficacy of said constructs in an *in vivo* system.

In another embodiment, the peptides of the present invention when complexed to the adeno-associated virus (AAV) Rep protein, can facilitate the integration of AAV DNA (and any DNA inserted into an AAV vector) into the genome of a target cell. The Rep protein of adeno-associated virus is able to facilitate integration of the AAV genome into a specific site on human chromosome 19. See Weitzman et al., *Proc. Natl. Acad. Sci. USA* 91:5808-5812 (1994). However the Rep protein is toxic and is difficult to deliver to cells as a DNA encoding the protein. In fact, to date it has not been feasible to generate a stable cell line constitutively expressing Rep or an adenoviral helper vector that can transiently express Rep. The present invention provides a complex comprising a peptide of the present invention (*e.g.* peptide 2, 3, 4 or 5) linked to the Rep protein. Such a complex facilitates the delivery of the Rep protein to a target cell. The target cell can be infected with AAV (or transfected with AAV DNA) before or after treatment with the peptide-Rep complex. The presence of the peptide-Rep complex and the AAV DNA allows for the

integration of the AAV DNA into the target cell genome.

The present invention is further directed to promoting the growth of defective viruses, such as HSV, in culture. The generation of defective viruses is useful for gene therapy applications. Defective viruses do not replicate without the help of necessary replication proteins which are not encoded by such defective viruses. One approach has been to construct cell lines expressing the necessary viral replication proteins (*e.g.* ICP0, ICP4, ICP22 and ICP27), which have been difficult to generate. In one embodiment of the present invention, replication defective virus is grown in cells by infecting the cells with the defective virus and administering one or more complexes comprising a peptide of the present invention linked to a protein necessary for the replication of the defective virus.

GST fusion proteins are widely used in research to study various proteins due to the ease of expressing and purifying such fusion proteins. The internalizing peptides of the present invention are useful for a universal system for delivering any GST fusion protein to cells. The GST fusion protein may be made by techniques known in the art, such as the method described by Pharmacia (Piscataway, NJ). The peptides of the present invention, when linked to glutathione, can facilitate the delivery of GST fusion proteins in a target cell. The glutathione-peptide constructs of the present invention can bind to any GST fusion protein and facilitate the internalization of the GST fusion protein into a cell (*see* Figure 21 and Example 8 below). The present invention is also directed to kits comprising the glutathione-peptide construct.

It is also an object of the present invention to provide an expression cassette comprising a nucleic acid encoding a fusion protein comprising a leader sequence, an internalizing peptide of the present invention, and a protein of interest, operably linked to expression control sequences. Such a fusion protein is capable of post-translational intercellular transport via the leader sequence or the internalizing peptides of the present invention. The leader sequence may be derived from secreted gene products such as interleukin-1 receptor antagonist (IL-1ra), Parathyroid hormone (PTH), or cathelin (*see* Huttner et al., *Ped. Res.* 45:785 (1999)). Since the leader sequence may be clipped or removed during translocation, the internalizing

peptides of the present invention ensure that the fusion protein encoded by the expression cassette of the present invention may still be internalized into surrounding cells even after removal of the leader sequences, thereby improving the efficiency of intercellular transport. The protein of interest may include, *inter alia*, apoptotic proteins, suicide proteins, therapeutic proteins, etc.. In addition, a herpes simplex virus protein, VP22, has been shown to be released from cells and taken up by neighboring cells. See Elliot & O'Hare, *Cell* 188:223-233 (1997); Elliot & O'Hare, *J. Virol.* 74:2131-2141 (2000); Derer et al., *J. Mol. Med.* 77:609-613 (1999). Another embodiment of the present invention is directed to a fusion construct comprising the leader sequence of VP22, a peptide of the present invention (preferably peptide 2, 3, 4 or 5) and a protein capable of achieving a desired effect in a cell (e.g. apoptotic protein, suicide protein, therapeutic protein, etc).

The expression cassette of the present invention may further comprise expression control sequences operably linked to the nucleic acid encoding the fusion protein and may be contained within a transfer vector which may be administered to cells either *in vivo* or *in vitro* and mediate expression therein. In addition to containing the DNA sequences encoding one or more transgenes, the chimeric adenoviral vectors of the invention may contain any expression control sequences such as a promoter or enhancer, a polyadenylation element, and any other regulatory elements that may be used to modulate or increase expression, all of which are operably linked in order to allow expression of the transgene. The use of any expression control sequences, or regulatory elements, which facilitate expression of the transgene is within the scope of the invention. Such sequences or elements may be capable of generating tissue-specific expression or be susceptible to induction by exogenous agents or stimuli. For example, suitable promoters include promoters such as from phosphoglycerate kinase (PGK) promoter or a cytomegalovirus (CMV). In one embodiment, a vector containing the expression cassette comprising DNA sequences encoding a fusion protein comprising a leader sequence, an internalizing peptide and a protein of interest is administered to a cell wherein said expression cassette is transcribed and translated and the resultant fusion protein is then secreted via the leader sequences. After secretion from the cell in which it was expressed, the

fusion protein comprising an internalizing peptide, therapeutic protein or other protein of interest, and optionally the leader sequence (which may alternatively be cleaved) may be internalized into surrounding cells *in vivo* or *in vitro* via the internalizing peptides of the present invention.

5 Such an expression cassette is useful for sustained delivery of a peptide-cargo complex in cells. Any leader sequence capable of directing the secretion of a polypeptide linked thereto is contemplated by the present invention, including, but not limited to IL-1ra, PTH and related sequences. The expression cassette comprising DNA sequences encoding a fusion protein comprising a leader
10 sequence, an internalizing peptide and a protein of interest is useful to direct the delivery of the protein of interest to surrounding cells. The protein of interest may be an apoptotic protein, anti-apoptotic protein, cell cycle regulatory protein, transcription factor, suicide gene product, viral or tumor antigens, or cell proliferation factors (*e.g.* viral oncoproteins, telomerase, etc.).

15 The invention is also related to methods of identifying the internalizing peptides of the present invention. Peptides having the ability to be internalized into cells can be identified by random peptide libraries coupled with an affinity enrichment process. A phage display peptide library kit, such as that supplied by New England Biolabs, Inc. (Beverly, MA) may be employed in the present invention for the
20 identification of peptides which are capable of being internalized into cells and are also capable of facilitating the internalization of cargo into cells. A random peptide library may also be presented on a plasmid (as part of a fusion protein) or protein as a peptide-protein complex by techniques known in the art. Methods of identifying internalizing peptides can facilitate the isolation of peptides with superior internalizing capabilities and
25 provide numerous peptides which can be selected for a reduced likelihood of eliciting an immune response when administered to a subject and an increased half life *in vivo* and/or *in vitro*.

 The method comprises (a) incubating a target cell with a peptide display library; (b) isolating internalized peptide presented by said peptide display library from
30 the cytoplasm and nuclei of the cells and identifying said peptides; (c) linking said peptides to cargo; (d) incubating said peptide-cargo complex with a target cell; and (e)

determining ability of said peptides to facilitate the uptake and, where desired, nuclear localization of said cargo into said target cell.

In a preferred embodiment, a random peptide library is presented on the surface of bacteriophage M13 as coat protein fusions creating a physical linkage between the displayed peptide and its encoding DNA sequence. *E.g.*, New England Biolabs, Inc. 5 Ph.D.TM phage display peptide library kits (Beverly, MA). Such phage display peptide libraries allow for the selection of peptide ligands for a variety of targets through biopanning, including panning against intact cells. *See* Barry and Johnston, 1996, *Nature Medicine* 2:299-305; Szardenings et al., 1997, *J. Biol. Chem.* 272:27943-27948. Panning 10 against intact cells may allow for the identification of peptides which facilitate the internalization of the phage on which they are displayed. *See* Vasily et al., 1999, *Biochimica et Biophysica Acta* 1448:450-462; and Vasily et al., 1999, *Biochimica et Biophysica Acta* 1448:463-472. Additionally, a T7 phage display library, which is able to express larger peptides fused to the carboxyl terminus of the T7 phage 10B fiber 15 protein (as compared to a 12 amino acid peptide library which is expressed on the coat of the New England Biolabs M13 library) may also be employed for biopanning.

The phage display peptide library may be incubated with a target cell line (e.g. Hig-82 cells) to isolate phage which are internalized into the cells (see Examples 2 and 3 below). The cells are then harvested and lysed to isolate the internalized phage 20 which express peptides which are capable of facilitating their internalization. The cell lysate is collected for phage titering and amplification in bacteria. The procedure is repeated with amplified phage a total of three times to obtain phage preparations which are enriched for the peptides responsible for the internalization of the phage. After three rounds of biopanning, titering and amplification, the phage are used to infect bacterial 25 lawns for the purpose of isolating single plaques representing a single peptide responsible for the internalization of the phage. The phage is then amplified and the phage DNA is isolated and sequenced to determine the sequence of the DNA encoding the peptide presented on the surface of the phage which was isolated by biopanning.

Where plasmid display library is used, random peptides are presented on 30 the surface of a plasmid according to U.S. Patent No. 5,338,665, incorporated herein by reference. The plasmid display library is then utilized in a manner similar to the method

employed for the phage display library by techniques known to those skilled in the art.

After determining the sequence of the peptides isolated by the biopanning (whether by phage or plasmid display libraries or any other technique known to those skilled in the art), "free" peptides (peptides without phage) may be synthesized according to peptide synthesis methods (e.g. Merrifield solid phase synthesis). Such peptides are then conjugated to cargo. In a preferred embodiment, the peptides are synthesized such that they are biotinylated and may be conjugated to avidin labeled cargo (e.g. avidin β -gal, avidin Cy3). This allows for ease of screening of multiple peptides for their ability to internalize cargo. Additionally, the peptide may be expressed as a fusion protein with the cargo of interest (e.g. β -gal) by methods known to those skilled in the art. See, e.g., Villaverde et al., 1998, *Biotechnology and Bioengineering* 59:294-301.

Other preferred cargo include, but are not limited to, proteins, such as suicide proteins (e.g. HSV TK), tumor suppressor proteins, transcription factors, kinase inhibitors, kinases, apoptotic proteins, anti-apoptotic proteins, cell cycle regulatory proteins, viral and cellular antigens, toxins, transgenes (encoding for, *inter alia*, protein, RNA, ribozymes, antisense RNA), RNA, plasmids, oligonucleotides (single and double stranded) and virus.

The peptide conjugates (peptide + cargo) are then incubated with a target cell to allow for delivery of the peptide-cargo complex into the cell (e.g. Hig-82 cells). The ability of the peptide to transfer the cargo into the target cell may be measured by the presence of the cargo in the target cell by techniques known in the art. Where the cargo is β -gal, the addition of Xgal to the cells will produce a blue color in the cells if the β -gal is present. Where the cargo is Cy3, confocal microscopy may be employed to determine whether the cells fluoresce. Functional assays may also determine the presence of cargo in a cell. For example, but not by way of limitation, where the cargo is CFTR (or a nucleic acid encoding CFTR), the manifestation of a functional chloride ion channel would indicate delivery of the CFTR cargo to the target cell. Where the cargo is a toxin, cell death may indicate the presence of the cargo in the target cell and, where the cargo is a virus (e.g. Human Immunodeficiency Virus, Murine Leukemia Virus, Equine Infections Anemia Virus), the virus may comprise green fluorescent protein (GFP) as a marker or the virus may be labeled with Cy3, also a fluorescent marker to track the

internalization of the virus by the peptides of the present invention in cells which would otherwise be resistant to infection by the virus. If the virus is a viral vector comprising a transgene, the presence of the virus in the cell may be demonstrated by the presence of a transgene product. The presence of the cargo in the nuclei by the methods described
5 above, indicates that the peptides are capable of facilitating the translocation of the cargo to the nucleus and may be demonstrated as described for internalization generally. For example, confocal microscopy may be used to demonstrate the presence of a fluorescent tagged molecule in the nucleus. Alternatively, the cells may be harvested and the nuclei separated therefrom for the determination of the presence of a functional cargo therein
10 by methods known to those skilled in the art.

In accordance with the present invention, screening for internalizing peptides by phage biopanning yielded the peptides represented by SEQ ID NO:1 through SEQ ID NO:18, further illustrated in Table 1 above. Particularly preferred peptides include KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3),
15 PIRRKCLRRLK (SEQ ID NO:4) and RRQRRTSKLMKR (SEQ ID NO:5) which facilitated the internalization of phage as well as the facilitation of the internalization of a cargo (e.g. β -gal and Cy3).

The peptides of the present invention may also be useful for the determination of the cell proteins which mediate internalization. For example, a cell
20 lysate may be prepared from the cells used to isolate the internalizing peptide. The internalizing peptide may be fused to a polypeptide (e.g. glutathione-S-transferase or poly-histidine) which can be used for immuno-affinity purification. The peptide fusion can then be incubated with the cellular lysate and passed over a column specific for the fusion peptide (e.g. a glutathione column for the glutathione-S-transferase fusion or a
25 nickel or cobalt column for the poly-his fusion). Proteins which bind to the internalizing peptide (e.g. cell surface receptors) may remain bound to the peptide fusion during the purification process and be purified along with the peptide fusion. The peptide-bound protein may then be isolated and its sequence may be determined by methods known in the art (e.g. N-terminal protein sequencing). Such determination may lead to the
30 identification of other pathways which might be useful for the delivery of cargo to a target cell.

The peptides of the present invention can be prepared by classical methods known in the art, for example, by using standard solid phase techniques. The standard methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis, and recombinant DNA technology.
5 See, e.g., Merrifield, 1963, *J. Am. Chem. Soc.* 85:2149, incorporated herein by reference.

On solid phase, the synthesis is typically commenced from the C-terminal end of the peptide using an alpha-amino protected resin. A suitable starting material can be prepared, for instance, by attaching the required alpha-amino acid to a chloroethylated resin, a hydroxymethyl resin, or a benzhydrylamine resin. One such chloromethylated
10 resin is sold under the tradename BIO-BEADS SX-1 by Bio Rad Laboratories, Richmond, CA, and the preparation of the hydroxymethyl resin is described by Bodonsky et al., 1966, *Chem. Ind.* 38:1597. The benzhydrylamine (BHA) resin has been described by Pietta and Marshall, 1970, *Chem. Commn.* 650 and is commercially available from Beckman Instruments, Inc., Palo Alto, CA, in the hydrochloride form.

15 Thus, the peptides of the invention can be prepared by coupling an alpha-amino protected amino acid to the chloromethylated resin with the aid of, for example, cesium bicarbonate catalyst, according to the method described by Gisin, 1973, *Helv. Chim. Acta.* 56:1467. After the initial coupling, the alpha-amino protecting group is removed by a choice of reagents including trifluoro acetic acid (TFA) or hydrochloric
20 acid (HCl) solutions in organic solvents at room temperature.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups urethane type protecting groups, aliphatic urethane protecting groups and alkyl type protecting groups. The side chain protecting group remains intact during coupling and is not split off during
25 the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. An excess of each
30 protected amino acid is generally used with an appropriate carboxyl group activator.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treatment with a reagent such as TFA or hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups.

5 These solid phase peptide synthesis procedures are well known in the art and are further described by Stewart and Young, *Solid Phase Synthesis* (2nd Ed., Pierce Chemical Co., 1984), incorporated herein by reference.

 The internalizing peptides of the present invention may be synthesized with additional groups, such as biotin or other markers, such that the peptide may be
10 tracked in the cell or conjugated via the additional group to cargo. The peptides may also be later modified to incorporate any desired additional groups according to methods known in the art.

 The internalizing peptides are typically synthesized as the free acid but could be readily prepared as the amide or ester where desired. Other types of
15 modifications include, but are not limited to, methylation, acetylation and adding a benzyloxycarbonyl (t-BOC) group. Additionally the peptides may be synthesized as cyclic peptides. The C-terminal carboxyl group or a C-terminal ester can be induced to cyclize by internal displacement of the -OH or ester of the carboxyl group or ester respectively with the N-terminal amino group to form a cyclic peptide. Such methods
20 are well known in the art.

 Cyclization of the peptides or incorporation of a desamino or descarboxy residue at the termini of the peptides of the present invention, so that there is no terminal amino or carboxy group, to decrease susceptibility to proteases or to restrict the confirmation of the peptide, are also contemplated by the present invention.

25 The present invention also provides for compositions comprising the internalizing peptides of the present invention, complexes comprising the peptides linked to cargo, and immunogens of the present invention. Non-limiting examples include: the administration of internalizing peptides and peptide-cargo *in vivo* by oral, pulmonary, parenteral (intramuscular, intra-articular, intraperitoneal, intravenous (IV) or
30 subcutaneous injection), inhalation (via a fine powder formulation or a fine mist), transdermal, nasal, vaginal, rectal, or sublingual routes of administration and can be

formulated in dosage forms appropriate for each route of administration.

The peptide-cargo complexes of the present invention may be administered with a carrier. Such carriers include any suitable physiological solution or dispersant or the like. The physiological solutions include any acceptable solution or dispersion media, such as saline or buffered saline. The carrier may also include antibacterial and antifungal agents, isotonic and adsorption delaying agents, and the like. Except insofar as any conventional media, carrier or agent is incompatible with the active ingredient, its use in the compositions is contemplated.

The invention is further directed to methods for using the compositions of the invention for *in vivo* or *ex vivo* applications in which it is desired to deliver cargo into cells to achieve a particular phenotypic effect. *In vivo* applications involve, *e.g.*, the direct administration of the peptide-cargo complex of the present invention formulated as a composition to the cells of an individual. *Ex vivo* applications involve, *e.g.*, the transfer of the peptide-cargo complex of the present invention directly to autologous cells which are maintained *in vitro*, followed by the re-administration of the cells comprising the internalized cargo to a recipient.

Dosage of the peptide-cargo complex of the present invention to be administered *in vivo* in order to effect efficient delivery of cargo into a target cell and/or achieve a phenotypic effect correlated to the delivery of cargo is determined with reference to various parameters, including the species of the subject, the age, weight, and disease status and the particular physiological conditions requiring phenotypic alteration. Dosage also depends upon the location of the cells to be targeted within the subject. For example, target cells of the lung may require different dosages than administration into the blood stream of an organism. The dosage is preferably chosen so that administration causes an effective result, as measured by molecular assays or phenotypic alteration. Such assays include Western blot of a particular protein being administered or encoded by a transgene that has been administered, immunoprecipitation, immunocytochemistry, or other techniques known to those skilled in the art. Dosages may range from 0.01 nM to 1 μ M. In a preferred embodiment, the dosage ranges from 1 nM to 1 μ M. In a particularly preferred embodiment, the dosage is 1.5 nM for pep4 and pep5 and 15 nM for pep2 and pep3.

The practice of the present invention can be achieved by employing a number of conventional techniques of molecular biology, microbiology, recombinant DNA technology, biochemistry and immunology which are within the skill of the art. Such techniques are explained fully in the literature, see, e.g., Fritsch and Maniatis, 5 *Molecular Cloning: A Laboratory Manual*, 2nd Edition (1989); Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, (1992); incorporated herein by reference.

Although only preferred embodiments of the invention are specifically described above, it will be appreciated that modifications and variations of the invention are possible without departing from the spirit and intended scope of the invention.

10 The following examples are provided to more clearly illustrate the aspects of the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1: Titering M13 Phage

A phage display library (Ph.D.-12™ Catalog # 8110) was obtained 15 from New England BioLabs (Beverly, MA). The Ph.D.-12™ phage display library is a library of M13 coliphage with each phage displaying a different 12 residue peptide and represents 1.9×10^9 independent clones. The randomized peptides in the library are expressed between the leader sequence and the N-terminus of the minor coat protein pIII, resulting in an average valency of 5 displayed peptides per virion. The 20 display vector for the library is a derivative of wild-type M13 phage which is not a lytic phage. There is a physical linkage between each displayed peptide and its encoding DNA for easy determination of the selected peptide sequence.

E. coli ER2537 was the host strain used for the M13 phage display library. ER2537 is a robust F+ strain with a rapid growth rate and is well suited for 25 M13 propagation.

For titering the phage, ER2537 was streaked out from a glycerol stock onto a minimal plate (500 ml 2X M9 salts (12 g Na_2HPO_4 , 6 g KH_2PO_4 , 1 g NaCl, 2 g NH_4Cl per liter), 500 ml 3% agar, 20 ml 20% glucose, 2 ml 1 M MgSO_4 , 0.1 ml 1 M CaCl_2 , 1 ml thiamine (10 mg/ml)) for phage titering. After 24 hours, a single colony 30 was picked and used to inoculate 5 ml of LB (10 g bacto tryptone, 5 g yeast extract,

and 5 g NaCl per liter) which was grown for 3 hours to mid-log phase ($OD_{600} \sim 0.5$) at 37°C . Approximately 200 μl of the stock was then spread onto a plate containing LB, IPTG and Xgal (per liter LB add 15 g agar, 0.05 g IPTG and 0.04 g Xgal). The plates were inoculated with serial dilutions of 10-fold of the phage stock and
5 incubated overnight at 37°C . The cells infected with phage stained blue due to the presence of the phage (which contains $\beta\text{-gal}$) and the plaques were counted to determine the titer. The titer was preferably $1\text{-}2 \times 10^{11}$. Biopanning (as described below in Example 2) can be carried out with as little as 10^9 plaque forming unites (pfu).

10 Example 2: Screening a Phage Display Library to Identify Internalizing Peptides:

Hig-82 biopanning: Hig-82 cells (rabbit synovial cell line supplied by Christopher Evans, University of Pittsburgh, ATCC Deposit No. CRL-1832) were employed for screening the New England Biolabs Ph.D-12™ phage-display library. The Hig-82 cells were cultured in 10 cm plates and grown to 100% confluency. The
15 cells were then incubated with approximately 4×10^{10} phage in a volume of 10 μl overnight at 4°C . The Hig-82 cells were then harvested and washed twenty times with wash buffer (25 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM CaCl_2 , 10 mM MgCl_2 , 1% bovine serum albumin (BSA)). The last washing solution was collected and titered to determine if any phage were present. This wash had no phage
20 indicating that the washing was sufficient. Phage which were bound to the cells were eluted with 50 mM glycine, pH 2.2 for 30 minutes at room temperature and the eluate was immediately thereafter neutralized for two minutes with 0.2M NaPO_4 buffer, pH 8.0. The eluate was collected for phage titering and amplifying.

The cells were then trypsinized from the plate with 0.05% trypsin, 0.53
25 mM EDTA and lysed by three consecutive rounds of freeze/thaw in dry ice/EtOH. The lysed cells were then centrifuged and the supernatant was collected for phage titering and amplification. The cell pellets containing the cell debris were washed with wash buffer (see above) five times and the last wash was collected for phage titering. The cell pellet containing the cell debris was then eluted with 50 mM
30 glycine, pH 2.2 for 30 minutes at room temperature and the eluate was immediately

thereafter neutralized for two minutes with 0.2 M NaPO₄ buffer, pH 8.0. The eluate was collected and saved for phage titering and amplification.

Phage titering was accomplished as described above in Example 1 and the phage were amplified by adding the eluates to a 20 ml ER2537 culture grown to early-log phase in LB medium as described above in Example 1 and incubating for 4.5 hours at 37 °C with vigorous shaking. The culture was then centrifuged for 10 minutes at 10,000 rpm in a Sorvall model SS-34 centrifuge at 4 °C. The supernatant was transferred to a new tube and spun a second time. The upper 80% of the supernatant was then transferred to a new tube and 1/6 volume of PEG/NaCl (20% w/v polyethylene glycol-8000, 2.5 M NaCl) was added and incubated overnight at 4 °C to precipitate the amplified phage. The PEG precipitate was then centrifuged for 15 minutes at 10,000 rpm at 4 °C (supernatant was decanted and pellet was respun briefly) and residual supernatant was removed with a pipette. The pellet was resuspended in 1 ml TBS (50 mM Tris-HCL (pH 7.5), 150 mM NaCl) and spun in a microcentrifuge tube to remove any remaining debris. The supernatant was transferred to a fresh microcentrifuge tube and re-precipitated with 1/6 volume PEG/NaCl, incubated for 60 minutes on ice and microcentrifuged for 10 minutes at 4 °C. The pellet was resuspended in 200 µl TBS, 0.02% NaN₃ and recentrifuged to remove any remaining debris. The supernatant represented the amplified phage.

The procedure (referred to hereafter as biopanning) was repeated a total of three times to achieve phage stocks enriched for phage which were internalized into the Hig-82 cells.

Human Primary T-cell biopanning: Human primary CD4⁺ and CD8⁺ T-cells (purified from peripheral blood mononuclear cells (PBMC) of normal donors using immunomagnetics beads (Miltenyi Biotech, Bergish Gladbach, Germany)) were employed for screening the New England Biolabs Ph.D-12™ phage-display library. The T-cells were incubated at 37°C overnight in the presence of 25 IU/ml interleukin2 (IL2). The cells were then incubated with approximately 4×10^{10} phage in a volume of 10µl for 4 hours at 4 °C with gentle shaking. The T-cells were then harvested and washed extensively with tris-buffered saline (TBS). Phage which were bound to the cells were eluted with 50 mM glycine, pH 2.2 for 10 minutes at room

temperature and the eluate was immediately thereafter neutralized for two minutes with 0.2M NaPO₄ buffer, pH 8.0. The eluate was collected for phage titering and amplifying.

5 The cells were then trypsinized from the plate with 0.05% trypsin, 0.53 mM EDTA, washed 2x with TBS at room temperature, centrifuged to remove wash and resuspended in 0.2 ml TBS. The T-cells were then lysed by three consecutive rounds of freeze/thaw in dry ice/EtOH. The lysed cells were then centrifuged and the supernatant was collected for phage titering and amplification.

10 Phage titering was accomplished as described above in Example 1 and the phage were amplified as described for Hig-82 cells.

The procedure (referred to hereafter as biopanning) was repeated a total of three times to achieve phage stocks enriched for phage which were internalized into the T-cells.

15 Calu 3 cell biopanning: The human lung adenocarcinoma cell line Calu 3 (ATCC, Rockville, MD.) was cultured in a flask with a 1:1 ratio of DMEM media and F12 media to 70% confluency, then trypsinized from the flask, washed 1x with TBS and transferred into a cell culture filter and grown to 100% confluency in a 1:1 ratio of DMEM media and F12 media. Phage biopanning was performed as above for human primary T-cells.

20 Cervical Tissue biopanning: Surgically resected cervical mucosa cells from human patients were grown in a 60 mm tissue culture dish in the presence of 5 ml of complete DMEM media. The cervical mucosa cells were then incubated with approximately 4×10^{10} phage in a tissue culture dish for 18 hours. The mucosa tissue was then trypsinized and class II positive cells were selected from a single cell
25 suspension using immunomagnetics beads (Miltenyi Biotech, Bergish Gladbach, Germany) following the manufacturer's protocol. The purified mucosal cells were then lysed by three consecutive rounds of freeze/thaw in a -70°C freezer. The lysed cells were then centrifuged and the supernatant was collected for phage titering and amplification.

30 Phage titering was accomplished as described above in Example 1 and the phage were amplified as described for Hig-82 cells.

Example 3: Identification of phage displayed peptides which were internalized into Hig-82 cells, T-cells, Calu3 cells, and Cervical Tissue.

After three rounds of biopanning, the enriched phage preparations were
plaqued as described above in Example 1 for phage titering. A single plaque was then
5 picked (from plated containing approximately 100 plaques) with a sterile wooden
stick and transferred to a tube containing 1 ml of ER2537 culture in LB and incubated
for 4.5 hours with shaking. The phage were amplified as described above in Example
2. Phage DNA was prepared from the amplified stock by centrifuging the 1 ml
cultures in a microcentrifuge for 30 seconds, removing the supernatant, adding 200 μ l
10 PEG/NaCl and precipitating the phage for 10 minutes at room temperature. The
precipitated phage were then centrifuged for 10 minutes in a microcentrifuge and the
supernatant was discarded (a subsequent spin was performed to remove any remaining
supernatant). The pellet was resuspended in 100 μ l iodide buffer (10 mM Tris-HCl
(pH 8.0), 1 mM EDTA, 4 M NaCl), 250 μ l EtOH was added and the mixture was
15 incubated for 10 minutes at room temperature to preferentially precipitate single-
stranded phage DNA and leave most of the phage protein in solution. The
precipitated phage DNA was then centrifuged for 10 minutes in a microcentrifuge and
the pellet was washed with 70% EtOH and dried briefly under vacuum. The dried
phage DNA pellet was then resuspended in 30 μ l TE buffer (10 mM Tris-HCl (pH
20 8.0, 1 mM EDTA). The phage DNA (approximately 5 μ l of the 30 μ l preparation)
was then sequenced (automated DNA sequencing at the University of Pittsburgh) to
determine the sequence of the peptides which were internalized. Figure 7 shows the
uptake of M13 phage labeled with Cy3 by peptide 3 (SEQ ID NO:3) and peptide 5
(SEQ ID NO:5). The peptides identified are pep 1 through pep 66 (SEQ ID Nos:1-18
25 and 25-72 respectively) as represented in Table 1-4 above.

To show that the peptides of the present invention are able to facilitate
internalization and transport of protein complexes and phage (virus) to the nucleus of
a cell, biotinylated peptides were coupled to streptavidin-488. See Bayer et al.,
Histochem. Cytochem. 24:933-939 (1976); Ivanenkov & Menon, *Biochim. Biophys.*
30 *Acta.* 1448:463-472 (1999). The use of the 488 fluorescent marker allows for analysis
of the treated cells by confocal microscopy to determine the exact location of the

complexes in the cells. Peptide 5 (SEQ ID NO:5) was able to facilitate the efficient internalization of the streptavidin-488 complex (Figure 13A) with a significant percentage of the peptide-streptavidin-488 complex being found in the nucleus by confocal microscopy of treated cells (Figure 13B).

5 To determine if the peptides of the present invention are able to facilitate internalization of intact M13 phage(a virus), as suggested by the screening and isolation procedure, a peptide 5/M13 phage complex was labeled with Cy3 (as for Figure 5) and added to rabbit synovial fibroblasts. Labeled peptide 5/M13 phage complex was detected in the treated cells (Figure 13D) compared to the control phage
10 (Figure 13C). Thus peptide 5, as well as peptides 2, 3, and 4 (SEQ ID NOs:2-4 respectively) were able to facilitate internalization and nuclear localization of protein complexes as well as intact M13 phage. Therefore, the peptides of the present invention are useful for facilitating the internalization of virus and viral vectors.

Example 4: Identifying peptides which facilitate internalization of conjugated cargo
15 (β -galactosidase and Cy3)

Internalizing peptides 1-6 (SEQ ID Nos:1-6 respectively), which were identified as described above in Example 3, were synthesized, and biotinylated (synthesis and biotinylation performed by the Biotech Center of the University of Pittsburgh). The biotinylated peptides were then conjugated to avidin β -gal or avidin
20 Cy3 at room temperature for 2 hours.

The peptide conjugates were then added to cells (Hig-82 cells, rabbit synovial cells, human synovial cells, rabbit synovial lining, human primary airway cells HBE 144, human primary islet cells, murine myoblast cells C2C12, dog kidney epithelial cells MDCK, murine fibroblast cells NIH3T3 and murine tumor cells MCA
25 205 (human synovial cells and human primary airway cells (HBE144) were isolated from patients from the Presbyterian Hospital, University of Pittsburgh by standard techniques for establishing a primary cell culture; human islet cells were provided by the University of Miami and were isolated by standard techniques for establishing a primary cell culture; C2C12, MDCK, 3T3 and MCA205 cells were purchased from
30 ATCC, Bethesda, Md.; all cells were cultured in DMEM and grown to 100%

confluency) and incubated while gently rotating at 37 °C for 3 hours with TBS buffer. The cells were then washed 10 times with TBS buffer and fixed with 4% paraformaldehyde at room temperature for 30 minutes. Cells were then washed 3 times with TBS buffer and stained with 1 mg/ml X-gal (Boehringer Mannheim, Indianapolis) at 37°C overnight for β -gal-conjugated peptides. Cells which were blue indicated the presence of β -gal in the cells. For Cy3-conjugated peptides, fluorescence of the cells as measured by confocal microscopy indicated the ability of the peptide to internalize the Cy3 (a small molecule cargo).

Figure 1A shows the results of peptide- β -gal complex internalization into Hig-82 cells using peptide 1, 2, 3, 4, 5 and 6 (SEQ ID Nos:1-6 respectively) as compared to antennapedia peptide (RQIKIWFQNRRMKWKK; SEQ ID NO:19), TAT-PTD (YGRKKRRQRRR; SEQ ID NO:21), and a random control peptide (ARPLEHGSDKAT; SEQ ID NO:20). Figure 1B shows the relative strength of internalization mediated by peptides 2, 3, 4 and 5 (SEQ ID NOs:2-5 respectively) as compared to antennapedia peptide (SEQ ID NO:19), TAT-PTD (SEQ ID NO:21), and a random control peptide (SEQ ID NO:20). In Figure 1B, various concentrations of peptides linked to β -gal were tested (1:1 = 150 nM of β -gal). The results show that peptides 4 and 5 (SEQ ID NOs:4 and 5 respectively) effect internalization of β -gal linked thereto at a concentration equal to or less than TAT-PTD, suggesting that internalization mediated by peptides 4 and 5 is equal or superior to internalization mediated by TAT-PTD. Peptides 3 and 4 achieved internalization of β -gal at a slightly higher concentration. Strikingly, peptides 2-5 were more effective than the commercially available antennapedia peptide.

Figure 2 A-D shows the ability of peptide 1 (SEQ ID NO:1) (Figure 2A and 2C, low and high magnification respectively) and peptide 3 (SEQ ID NO:3) (Figure 2B and 2D, low and high magnification respectively) to facilitate the internalization of β -gal into rabbit synovial cells. Figure 3A-D shows the ability of peptide 5 (SEQ ID NO:5) (Figure 3A and 3B, high and low magnification respectively) and peptide 1 (SEQ ID NO:1) (Figure 3C and 3D, high and low magnification respectively) to facilitate the internalization of β -gal into human synovial cells.

Figure 4 shows the ability of peptides 2- 5 (SEQ ID NOs:2-5, respectively), TAT-PTD (SEQ ID NO:21), antennapedia peptide (SEQ ID NO:19), a random control peptide (SEQ ID NO:20) and an adenoviral vector encoding β -gal (Ψ 5) to facilitate *in vivo* transfer of peptide-conjugated β -gal into rabbit synovial lining. Peptide- β -gal complexes (fusions) were injected into rabbit knee synovial lining, the lining was then removed, washed with wash buffer, fixed with 4% paraformaldehyde, and stained with X-gal. Peptides 4, 5 and TAT-PTD showed the highest level of uptake into the rabbit synovial lining (Figure 4A). The control peptide and saline alone showed no uptake. The level of uptake was significantly higher for all the internalizing peptides as compared to the adenoviral vector encoding β -gal, which was injected 3 days prior to injection of the peptide- β -gal constructs (Figure 4A).

Figure 4B shows the histological analysis of the rabbit synovial lining using an eosin counter-stain. The histological analysis showed that β -gal staining was intracellular and limited to the synovial lining. Peptide 5 (SEQ ID NO:5) was able to facilitate uptake of β -gal in nearly 100% of the synovial cells *in vivo* (Figure 4B). Injection of peptide 5 (SEQ ID NO:5) into day 14 murine tumors which were prepared by subcutaneously injecting MCA-205 cells (fibrosarcoma cell line) resulted in significant β -gal staining that was also significantly higher than that observed for an adenoviral vector encoding β -gal. These *in vivo* results show that the peptides of the present invention can facilitate efficient internalization of protein complexes into joints and tumors and thus are useful for delivery of proteins of interest (such as apoptotic proteins, suicide proteins, tumor suppressor proteins, chemotherapeutic agents, etc) to joints (*e.g.* arthritic joints) and tumor cells.

Figure 6 A-I shows the ability of peptide 5 (SEQ ID NO:5) to facilitate the uptake of β -gal in (6A) HIG-82 cells; (6B) rabbit primary synovial cells; (6C) human primary synovial cells; (6D) HBE 144 primary human airway epithelial cells; (6E) MDCK polarized canine kidney cells; (6F) human β islet primary cells; (6G) C2C12 murine myoblast cells; (6H) MCA205 murine fibrosarcoma cells; and (6I) NIH3T3 cells. Additionally, Figure 9B-C shows the ability of peptide 5 to facilitate internalization of eGFP in human islets at low magnification (9B is a

photomicrograph of the histological ly stained cells and 9C shows the fluorescent detection of eGFP). Figure 9D-E show the ability of peptide 5 to facilitate internalization eGFP in human islets at high magnification (9D is a photomicrograph of the histological ly stained cells and 9E shows the fluorescent detection of eGFP).
5 Figure 9F-G shows the ability of peptide 5 to facilitate the internalization of eGFP in human dendritic cells (9F is a photomicrograph of the histological ly stained cells and 9G shows the fluorescent detection of eGFP). Figure 9A is a schematic representation of the expression construct encoding the peptide5-eGFP fusion protein.

Figure 17 shows the ability of peptide-5 linked to β -gal to transduce
10 CD34+/LIN- stem cells. Peptide 5 is biotin labeled and the β -gal is avidin labeled so that they may be linked together by an avidin/biotin interaction. The cells which stained dark were transduced with the peptide/ β -gal complex as indicated by an arrow.

To make the nucleic acid encoding the peptide-eGFP fusion depicted
15 in Figure 9A, PCR was employed using 5' and 3' primers that encoded the peptide and a his tag, respectively, and had complimentary sequences to eGFP DNA coding sequences. The resultant PCR product was subcloned into the bacterial expression vector pET ((Novagen, Madison, WI) . For protein expression, the expression vector comprising the DNA encoding a peptide eGFP fusion with a his tag was transfected
20 into bacterial cells which were grown by standard techniques. The cells were induced to express the fusion product with 1 mM IPTG and were harvested and lysed. The fusion protein was purified over a Nickel column. The purified protein was then added to the media of Hig-82 cells in culture. The eGFP positive cells were visualized under a fluorescence microscope. Figure 8 shows the ability of peptide 3
25 (SEQ ID NO:3) and peptide 5 (SEQ ID NO:5), as well as the antennapedia peptide (SEQ ID NO:19) to facilitate the internalization of β -gal into murine tumor cells *in vivo*. MCA 205 tumor cells (5×10^5 cells) were injected subcutaneously into the flank of a C57/BL6 mouse. At day 14, a single intra-tumoral injection of the peptide β -gal complex was performed. The mice were sacrificed 3 hours post-injection and the
30 tumor tissue was isolated, sectioned and stained with X-gal.

These data indicate that the internalizing peptides of the present invention can facilitate the uptake of cargo (β -gal or Cy3) into cells both *in vitro* and *in vitro*. Additionally, since the cargo was transported to the nucleus of the cells, the data also indicate that the peptides facilitated nuclear translocation of the cargo as well (see Figures 1, 2, 3, and 6).

Example 5: Peptide competition assay

A peptide competition assay was carried out to determine the relative efficiency and specificity of the various peptides. The peptides were conjugated to β -gal as described above in Example 4 and incubated with Hig-82 cells grown to 85% confluency in 24 well plates in the presence of non biotinylated peptides for 3 hours at 37 °C in TBS buffer while being gently rotated. The cells were then washed 10 times with TBS buffer, fixed with 4% paraformaldehyde at room temperature for 30 minutes, washed 3 times with TBS buffer, and stained with Xgal, as described above in Example 4, overnight. Cells which stained blue had internalized peptide-conjugated β -gal.

Figure 5 shows the ability of the various non-biotinylated peptides to compete for internalization of the peptide-conjugated β -gal. In rows 1-4 peptide-conjugated β -gal comprising peptide 2, 3, 4 and 5 respectively (SEQ ID Nos: 2-5 respectively) were incubated with cells in the presence of non biotinylated peptides 1-6 (SEQ ID Nos:1-6 respectively) which were added to columns 1-6 respectively. Peptide 1 (SEQ ID NO:1) did not block the internalization by any of the peptides. Peptides 2-6 (SEQ ID Nos:2-6 respectively) blocked the internalization of peptide 2-conjugated β -gal and peptides 4 (SEQ ID NO:4) and peptide 5 (SEQ ID NO:5) blocked the internalization of peptide 2 through peptide 6-conjugated β -gal indicating that these peptides more efficiently bound to the cells than any others.

Figure 12 also shows the ability of the various non-biotinylated peptides to compete for internalization of the peptide-conjugated β -gal. A 100 fold excess of peptide 5 (SEQ ID NO:5) was able to completely inhibit the uptake of peptides 3, 4 (SEQ ID NOs:3 and 4 respectively) and the antennapedia peptide (SEQ ID NO:19) and significantly inhibit the uptake of TAT-PTD (SEQ ID NO:21) and

itself. Surprisingly peptide 6 (SEQ ID NO:6) was able to inhibit the uptake of peptide 2 and 3, but not peptides 4 and 5, even though peptide 6 is only weakly able to transduce cells compared with peptides 2-5. Also tested were saline alone, a random peptide (SEQ ID NO:20), TAT-PTD (SEQ ID NO:21) and a polylysine peptide
5 having a MW between 1,000 D and 4,000 D with an average MW of 2,500 D (purchased from Sigma, St. Louis, MO). The ability of the polylysine peptide to inhibit uptake suggests that the charge of the peptide is important for at least part of the process of peptide-mediated internalization.

Example 6: Circular Dichroism Spectroscopy:

10 Stock solutions of peptides 1, 2, 3, 4, 5 and 6 (SEQ ID NOs:1-6 respectively), as well as TAT-PTD (SEQ ID NO:21), and the antennapedia peptide (SEQ ID NO:19) were diluted to 1.5 mg/ml in 5 mM KPO₄ at pH 7.4. Measurements were made using an Aviv 62DS spectropolarimeter over the wavelength range from 300 to 190 nm (except for TAT-PTD, where sample absorbency precluded
15 measurements below 192 nm even after sample dilution), with a 0.5 nm step size, at room temperature using a 0.1 cm pathlength quartz cell (Hellma). Ellipticity measurements below 190 nm were precluded due to solvent absorption. All reported spectra were baseline corrected (by subtraction of similarly collected, averaged baselines of buffer alone) and smoothed using a Savitzky Golay filter.

20 Figure 10A-H shows that the peptides have a small dip at a wavelength of 225 nm which indicative of possible insignificant secondary structure for α helices.. Specifically, Figure 10 A-H shows the circular dichroism plot for peptide 1 (SEQ ID NO:1; Figure 10A), peptide 2 (SEQ ID NO:2; Figure 10B), peptide 3 (SEQ ID NO:3; Figure 10C), peptide 4 (SEQ ID NO:4; Figure 10D),
25 peptide 5 (SEQ ID NO:5; Figure 10E), peptide 6 (SEQ ID NO:6; Figure 10F), antennapedia peptide (SEQ ID NO:19; Figure 10G) and a random peptide (SEQ ID NO:20; Figure 10H).

Figure 11A & B shows that qualitatively, the spectra of peptides 1-6 fall into three general groups. Figure 11A shows that the spectra of the highly active
30 peptides 4 and 5 (SEQ ID NOs: 4 and 5 respectively) are nearly super-imposable with

that of TAT-PTD (SEQ ID NO:21) and somewhat similar to the antennapedia peptide (SEQ ID NO:19). Peptides 2 and 3 (SEQ ID NOs: 2 and 3 respectively), which have an intermediate activity, yield spectra which are similar to each other, but significantly different from peptides 4 and 5. The lower activity peptides 1 and 6 (SEQ ID NOs: 1 and 6 respectively) fall into a third class and share some similarity to the random peptide (SEQ ID NO:20) which does not have activity. The peptides do not have a significant helical content, which would give rise to a bilobed minima at ~205 and 220 nm, and a large positive peak at ~195 nm. Rather, the peptides appear to be enriched in a poly-proline-type helix (which does not require the presence of prolines). See Sreerama & Woody, *Biochemistry* 33:10022-10025 (1994). This type of structure is consistent with some of the observations made regarding the antennapedia peptide where the substitution of the prolines into the peptide sequence did not disrupt activity, nor did the substitution of D-amino acids and/or reversal of the chain direction disrupt activity. See Derossi et al., *J. Biochem.* 217:18188-18193 (1996); Berlose et al., *Eur. J. Biochem.* 242:372 (1996). These substitutions would not affect the charge distribution on a poly-proline-type helix, nor be disruptive to this structure. The amphipathic cationic peptides of the present invention likely interact with the negative charges on the surface of the bilayers.

The CD spectra of the peptides was also analyzed following addition to small unimellar vesicles (SUVs). In the presence of SUVs, which comprise mixed phospholipids that are primarily phosphatidylcholines, no significant changes were observed in the CD spectra. However, when the SUVs were composed of dipalmitoyl phosphatidylserine, a large change in the resulting CD spectra was observed which correlated with transfer activity.

Example 7: Delivery of an Apoptotic Peptide to Cells via the Peptides of the Present Invention:

To demonstrate that the peptides of the present invention could facilitate the uptake of an apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) into cells and induce apoptosis therein, a continuous peptide, termed the "death peptide", comprising peptide 5 and the apoptotic peptide was synthesized

(RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24)) (Research Genetics, Huntsville, AL).

2 x 10⁵ cells (HIG82, MCA205 or Jurkatt) were seeded into 24 well plates. Forty-eight hours later, 100 μ M, 50 μ M, 25 μ M, 6.25 μ M and 3.1 μ M of the
5 either the death peptide (SEQ ID NO:24), peptide 5 alone (SEQ ID NO:5) or the apoptotic peptide alone (SEQ ID NO:23) was added to the cells. To check for cell viability, three hours later, the media was aspirated and 1 ml of serum-free media containing 0.25 mg of MTT was added to each well and incubated for 4 hours at 37°C. MTT-containing media was then removed and 1 ml of absolute isopropanol
10 containing 0.4N HCl was added. Photographs were taken of the plates, then the cells were harvested and any debris was centrifuged. OD was taken at λ = 570 nm. Each sample point was performed in triplicate.

Figure 14 and Figure 15 shows the ability of the "death peptide" to impair HIG-82 cell viability. In contrast, peptide 5 alone and the apoptotic peptide
15 alone did not impair viability.

To determine whether the death peptide could induce apoptosis in tumor cells and inhibit tumor growth *in vivo*, C57BL/6 mice bearing day 7 tumors in each flask (seeded with 1 x 10⁵ MCA205 cells) were injected daily for 10 days with a 50 μ l volume of 1 mM death peptide (SEQ ID NO:24; DP1) or the apoptotic peptide
20 alone (SEQ ID NO:23; KLA) or a saline mock into both tumors. Five mice were used in each group. Tumor volume was estimated by multiplying maximum length x width². Separately, C57BL/6 with single, day 12 tumors were injected with 1 mM death peptide (SEQ ID NO:24; DP1), apoptotic peptide alone (SEQ ID NO:23; KLA) or saline for eleven days. Ten mice comprised each group. On the final day, the mice
25 were injected with the appropriate saline or peptide solution and sacrificed 3 hours post-injection. Tumors were paraffin-embedded, sectioned, and stained for TUNEL and counterstained with methyl green or stained with hematoxylin and eosin to reveal histologic architecture.

As shown in Figure 16A, daily administration of 50 μ l of 1mM death
30 peptide (SEQ ID NO:24; DP1) lead to a striking reduction or halt in tumor growth in the fibrosarcomas compared to the apoptotic peptide alone (SEQ ID NO:23; KLA) or

tris buffered saline (TBS). By day 6, between the death peptide (DP1) and apoptotic peptide alone (KLA) groups, a $p \leq 0.026$ by a two-tailed student's t-test of the means was observed, which became $= 0.0001$ by day 10. As a plot of individual tumor sizes shows (Figure 16D), both the death peptide (DP1) and the apoptotic peptide alone (KLA) mouse groups begin with comparable tumor sizes. However, by day 9 of treatment, no overlap exists in the tumor sizes of the DP1-treated cohort with KLA-treated cohort. To test whether an immune response against the tumors may have been triggered by the apoptosis, treatment was halted at day 14, however, in the DP1-treated mice. Tumors continued to grow, directly correlating with the cessation of DP1 administration. Figure 16B shows a comparison of tumor sizes between the DP1 and KLA-treated groups eight days following the initiation of treatment. There is a dramatic reduction in tumor volume in the DP1-treated mice, coincident with reduction in ulceration and bleeding in these mice. In a separate experiment, 3 out of 10 mice with single flank tumors treated with DP1 had undetectable tumors following 11 days of treatment. Importantly, repeated administration of DP1 resulted in no obvious side-effects in treated mice.

To test whether apoptosis was the mechanism of tumor killing *in vivo* and to study the effects of death peptide administration on tumor architecture, fibrosarcomas treated daily for 11 days with death peptide (SEQ ID NO:24; DP1), apoptotic peptide alone (SEQ ID NO:23; KLA) or tris buffered saline (TBS) were injected with 50 μ l of 1 mM DP1, KLA or TBS on the eleventh day and sacrificed three hours later. Tumors were excised, embedded, sectioned, and stained with either TUNEL or hematoxylin and eosin. As shown in Figure 16C, treatment with DP1 mediates a potent apoptosis in MCA205 fibrosarcomas *in vivo*. A broad zone of anuclear and acellular eosinophilic debris is ringed by a zone of tumor cells in the process of undergoing apoptosis, as shown in the bottom right panel (Figure 16C). The zones of killing are substantial; in some instances, an estimated 10-20% of total tumor volume stained TUNEL-positive following DP1 administration. No TUNEL staining is observed in the KLA-treated tumors, indicating the requirement for linkage to peptide 5 (SEQ ID NO:5) for induction of apoptosis.

In addition, the death peptide was administered to rabbits with IL-1-induced arthritis (*see Ghivizzani et al. J. Immunol. 159:3604 (1997)*). Three arthritic rabbits received the death peptide, three received peptide 5 alone and 3 received the apoptotic protein alone. The rabbits were sacrificed 24 or 72 hours post-injection of the peptides and the rabbit knee capsules were removed for histology analysis and TUNEL staining.

Figure 18 shows that the delivery of the death peptide (SEQ ID NO:24; DP1) mediated apoptosis of hyperplastic synovium *in vivo* whereas the antimicrobial peptide alone (SEQ ID NO:23; KLA) did not. Furthermore, injection of the death peptide (SEQ ID NO:24; DP1) into arthritic rabbit joints caused a great reduction in the number of white blood cells in the lavage fluid if IL-1 inflamed rabbit joints as compared to peptide 5 alone (SEQ ID NO:5; peptide control) (*see Figure 19*). These data indicate that the internalizing peptides of the present invention are effective for delivering apoptosis factors to arthritic joints and may be useful for the treatment of arthritis.

To demonstrate that other apoptotic factors could effectively be delivered to cells, the internalizing peptide 5 (SEQ ID NO:5; pep 5) of the present invention was linked to p53. An expression clone was prepared by using PCR to first create an expression cassette having pep5 at the amino terminus and a his tag at the carboxy terminus of the p53 coding sequence. The expression cassette was cloned into the pet3b vector (Stratagene, La Jolla, CA). The expression clone was then transformed into BL21 *E. coli* expression strain and induced with 0.5mM IPTG for fusion protein expression. The fusion protein was purified using a nickel column.

HIG-82 cells were grown as described above and transfected with a reporter plasmid expressing the luciferase gene driven by the p21 promoter. The HIG-82 cells were transfected with p21-luciferase plasmid by calcium phosphate methods. In addition, as positive controls, a CMV promoter driven p53 plasmid and an Adp53 viral vector were transfected into HIG-82 cells together with the reporter plasmid expressing the luciferase gene driven by the p21 promoter. Purified pep5/p53/his was added to the culture 6 hours post addition of the reporter plasmid expressing the luciferase gene driven by the p21 promoter.

The reporter plasmid expresses the luciferase gene when p53 binds to the p21 promoter. Therefore, the presence of p53 in cells transfected with the reporter plasmid may be monitored by the presence of luciferase activity in the cells. To check for the presence of luciferase activity in the cells, the cells were washed 2x with PBS, harvested and lysed. The cellular lysate was used in a luciferase activity assay performed using a luciferase assay kit (Promega, Madison, WI).

Figure 20 shows the ability of pep5:p53 to induce p21 promoter driven luciferase expression in HIG-82 cells. Pep5:p53 was able to induce luciferase expression to similar levels as a plasmid expressing p53 and was much more effective than an adenovirus vector expressing p53. These data indicate that the internalizing peptides of the present invention can effectively deliver and internalize p53 to cells.

Example 8: Facilitation of the Delivery of GST Fusion Proteins to Cells Using Glutathione- linked Internalizing Peptides of the Present Invention

GST-eGFP (glutathione-S-transferase tagged green fluorescent protein), having in addition a histidine tag, was expressed in *E. coli* and purified using conventional techniques using a Nickel column to which the histidine tag binds (see Mi et al., *Mol. Ther.* (2000) in press). The purified GST-eGFP (200 μ l of 0.8 mg/ml/ total of 0.16 mg in TBS containing 1mM CaCl₂ and 10 mM MgCl₂) was incubated together with 50 μ l pep5 (SEQ ID NO:5; 2 mg/ml in TBS) in a total volume of 500 μ l by rotating overnight at 4°C. The mixture was then dialyzed against TBS at 4°C for 2 hours with one change of buffer.

Hig-82 cells were grown between 80% to 100% confluency in 12 well plates. The cells were washed 2x with 1 ml of TBS containing 1mM CaCl₂ and 10 mM MgCl₂, and 0.1% BSA. After the final wash, various dilutions of the glutathione-pep5-GST-eGFP complex (4X, 10X, 20X) were added to the cells, as well as the negative control (GST-eGFP alone) and "enriched" TBS. The cells were incubated together with the complex or controls at 37°C for 2 hours. The cells were then washed with "enriched" TBS 3x and examined by fluorescent microscopy.

Figure 21 shows that the glutathione-pep5:GST-eGFP complex was very effectively internalized by HIG-82 cells (panel A) as compared to the GST-eGFP alone (panel B) indicating that glutathione linked internalizing peptides of the present

invention are useful for facilitating the uptake of GST proteins to target cells.

CLAIMS

1. A peptide having an amino acid sequence selected from the group
consisting of KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2),
5 PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4),
RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
RQSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10),
PSKRLHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12),
10 PQNRLQIRRHKS (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14),
SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26),
PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28),
15 SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30),
SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38),
20 FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40),
FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42),
SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44),
SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46),
SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48),
25 SSSIFPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50),
LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52),
VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
AIPNNQLGFPPK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56),
YPYDANHTRSPT (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58),
30 TLPSPLALLTVH (SEQ ID NO:59), HPGSPFPPEHRP (SEQ ID NO:60),

TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP (SEQ ID NO:62),
VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
5 SNPWDSLLSVST (SEQ ID NO:69), KTIEAHPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

10 2. The peptide of claim 1 wherein said peptide facilitates cellular
internalization of a cargo linked thereto.

 3. The peptide of claim 2 wherein the peptide has the amino acid
sequence KRIHPRLTRSIR (SEQ ID NO:2).

 4. The peptide of claim 2 wherein the peptide has the amino acid
15 sequence PPRLRKRRQLNM (SEQ ID NO:3).

 5. The peptide of claim 2 wherein the peptide has the amino acid
sequence PIRRRKKLRLK (SEQ ID NO:4).

 6. The peptide of claim 2 wherein the peptide has the amino acid
sequence RRQRRTSKLMKR (SEQ ID NO:5).

20 7. The peptide of claim 1 wherein the peptide provides for nuclear
translocation in a target cell.

 8. A peptide-cargo complex comprising a peptide and a cargo
wherein the peptide has an amino acid sequence selected from the group consisting of
KRIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2),

- PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKCLRRLK (SEQ ID NO:4),
RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10),
5 PSKRLHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12),
PQNRLQIRRHKS (SEQ ID NO:13), PPHNRIRRLNM (SEQ ID NO:14),
SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26),
10 PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28),
SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30),
SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
15 LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38),
FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40),
FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42),
SLGWMLPFSPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44),
SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46),
20 SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48),
SSSIFPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50),
LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52),
VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
AIPNNQLGFPEK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56),
25 YPYDANHTRSPT (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58),
TLPSPLALLTVH (SEQ ID NO:59), HPGSPFPPEHRP (SEQ ID NO:60),
TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP (SEQ ID NO:62),
VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
TPKMTMTQYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
30 QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSLLSVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),

EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72)
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

5 9. The peptide-cargo complex of claim 8 wherein the cargo is
selected from the group consisting of a polynucleotide, a polypeptide, a small
molecule, a virus, a modified virus, a viral vector, and a plasmid.

 10. The peptide-cargo complex of claim 9 wherein the cargo is a
virus selected from the group consisting of adenovirus, adeno-associated virus, herpes
10 simplex virus, and retrovirus.

 11. The peptide-cargo complex of claim 8 wherein the cargo is
selected from the group consisting of therapeutic proteins, suicide proteins, tumor
suppressor proteins, transcription factors, kinase inhibitors, kinases, cell cycle
regulatory proteins, apoptotic proteins, anti-apoptotic proteins, viral antigens, cellular
15 antigens, differentiation factors, immortalization factors and toxins.

 12. The peptide-cargo complex of claim 8 wherein the peptide
facilitates cellular internalization of cargo linked thereto.

 13. The peptide-cargo complex of claim 12 wherein the peptide
provides for nuclear translocation of said peptide-cargo complex in a target cell.

20 14. The peptide-cargo complex of claim 8 wherein the peptide has
the amino acid sequence KRIHPRLTRSIR (SEQ ID NO:2).

 15. The peptide-cargo complex of claim 8 wherein the peptide has
the amino acid sequence PPRLRKRRQLNM (SEQ ID NO:3).

16. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence PIRRRKLLRRLK (SEQ ID NO:4).

17. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence RRQRRTSKLMKR (SEQ ID NO:5).

18. The peptide-cargo complex of claim 8 wherein the peptide is biotinylated and the cargo is avidin labeled.

19. The peptide-cargo complex of claim 11, wherein the cargo is an apoptotic protein selected from the group consisting of p53, caspase-3, HSV thymidine kinase and an antimicrobial peptide.

20. The peptide-cargo complex of claim 19, wherein the antimicrobial peptide is selected from the group consisting of KLAKLAK (SEQ ID NO:22) and KLAKLAKKLAKLAK (SEQ ID NO:23).

21. The peptide-cargo complex of claim 19 wherein having an amino acid sequence RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24).

22. The peptide-cargo complex of claim 8 wherein the cargo is glutathione.

23. A method for identifying peptides capable of cellular internalization of cargo linked thereto, said method comprising:

- incubating a target cell with a peptide display library;
- isolating internalized peptides presented by said peptide display library from said target cells and identifying said peptides;
- synthesizing said peptides;
- linking said peptides to cargo to form a peptide-cargo complex;

incubating said peptide-cargo complex with a target cell; and
determining the ability of said peptide to facilitate the cellular
internalization of said cargo into said target cell.

24. The method of claim 23 further comprising determining the
5 ability of said peptide to facilitate the nuclear localization of said cargo.

25. The method of claim 23 wherein said peptide display library is
an M13 phage display library.

26. The method of claim 25 wherein said phage display library
comprises peptides which comprise 12 amino acids.

10 27. The method of claim 23 further comprising biotin labeling the
peptides during or after said synthesis of the peptides.

28. The method of claim 23 further comprising avidin labeling the
cargo.

15 29. The method of claim 23 wherein the cargo is selected from the
group consisting of a polynucleotide, a polypeptide, a small molecule, a virus, a
modified virus, a viral vector, and a plasmid.

30. An expression cassette comprising a DNA encoding a fusion
protein comprising a leader sequence, a protein of interest and an internalizing peptide
having an amino acid sequence selected from the group consisting of
20 KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2),
PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4),
RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
RQSRRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
SRRKRQRSNMRI (SEQ ID NO:9), QIRKSKISRTL (SEQ ID NO:10),

- PSKRLHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12),
PQNRLQIRRHRSK (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14),
SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
5 HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26),
PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28),
SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30),
SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
10 HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38),
FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40),
FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42),
SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44),
15 SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46),
SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48),
SSSIFPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50),
LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52),
VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
20 AIPNNQLGFPPK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56),
YPYDANHTRSPT (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58),
TLPSPLALLTVH (SEQ ID NO:59), HPGSPFPPEHRP (SEQ ID NO:60),
TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP (SEQ ID NO:62),
VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
25 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
30 GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

31. The expression cassette of claim 30 further comprising expression control sequences operatively linked to said DNA.

32. A transfer vector comprising the expression cassette of claim 31.

5 33. The expression cassette of claim 30, wherein said leader sequences are selected from the group consisting of Il-1ra, PTH, VP-22 and related sequences.

10 34. The expression cassette of claim 30 wherein the protein of interest is selected from the group consisting of apoptotic protein, anti-apoptotic protein, cell cycle regulatory protein, transcription factor, suicide gene product, viral or tumor antigens, and cell proliferation factors.

15 35. The expression cassette of claim 30, wherein the encoded fusion protein comprises an amino acid sequence which facilitates removal of leader sequences therefrom and wherein said leaderless fusion protein comprises an internalizing peptide and a protein of interest.

36. The expression cassette of claim 30 wherein said fusion protein encoded thereby is produced and secreted from a cell and subsequently internalized into surrounding cells.

20 37. A method for inducing synovial cell death comprising administering a peptide-cargo complex to said synovial cell.

38. A method for inducing apoptosis in a tumor cell comprising administering a peptide-cargo complex to said tumor cell.

39. A method for reducing white blood cells in arthritic joints comprising administering a peptide-cargo complex to said white blood cells.

40. The method of claim 37, 38 or 39 wherein the peptide has an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLLHNNLRR (SEQ ID NO:11),
5 HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHSHK (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRLIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),
15 LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),
20 FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
25 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54), AIPNNQLGFPFK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT (SEQ ID NO:57),
30 DPATNPGPHFPR (SEQ ID NO:58), TLPSPALLTVH (SEQ ID NO:59),

HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61),
MTPSSLSTLPWP (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63),
QPIIHTSPYLPS (SEQ ID NO:64), TPKTMTQTYDFS (SEQ ID NO:65),
NSGTMQSASRAT (SEQ ID NO:66), QAASRVENYMHR (SEQ ID NO:67),
5 HQHKPPPLTNNW (SEQ ID NO:68), SNPWDSL SVST (SEQ ID NO:69),
KTIEAHPYYAS (SEQ ID NO:70), EPDNWSLDFPRR (SEQ ID NO:71),
HQHKPPPLTNNW (SEQ ID NO:72), GVVGKLGQRRTKKQRRQKK (SEQ ID
NO:73), GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

10 41. The method of claim 37, 38 or 39 wherein the cargo is an
apoptotic protein.

42. The method of claim 37, 38 or 39 wherein the apoptotic protein
is selected from the group consisting of p53, caspase-3, HSV thymidine kinase and an
antimicrobial peptide.

15 43. The method of claim 41 wherein the antimicrobial peptide has
an amino acid sequence selected from the group consisting of KLAKLAK (SEQ ID
NO:22) and KLAKLAKKLAKLAK (SEQ ID NO:23).

20 44. The method of claim 37, 38 or 39 wherein the peptide-cargo
complex has an amino acid sequence RRQRRTSKLMKRGGKLAKLAKKLAKLAK
(SEQ ID NO:24).

45. A method for internalizing a GST-fusion protein into a cell
comprising administering to said cell a peptide-cargo complex and a GST fusion
protein.

25 46. The method of claim 45 wherein the peptide has an amino acid
sequence selected from the group consisting of KRRIQRILSRNS (SEQ ID NO:1),

- KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3),
PIRRRKKLRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5),
MHKRPTTPSRKM (SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7),
RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9),
5 QRIRKSKISRTL (SEQ ID NO:10), PSKRL LHNNLRR (SEQ ID NO:11),
HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHSH (SEQ ID NO:13),
PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15),
GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17),
NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25),
10 AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),
LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29),
YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31),
AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33),
QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35),
15 HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPV LW (SEQ ID NO:37),
FQPYDHAEVSY (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),
FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
20 SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51),
LPHPV LHMGPLR (SEQ ID NO:52), VSKQPYYMWNGN (SEQ ID NO:53),
NYTTYKSHFQDR (SEQ ID NO:54), AIPNNQLGF PFK (SEQ ID NO:55),
25 NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT (SEQ ID NO:57),
DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61),
MTPSSLSTLPWP (SEQ ID NO:62), VLGQSGYLMPPMR (SEQ ID NO:63),
QPIIITSPYLPS (SEQ ID NO:64), TPKTMTQTYDFS (SEQ ID NO:65),
30 NSGTMQSASRAT (SEQ ID NO:66), QAASRVENY MHR (SEQ ID NO:67),
HQHKPPPLTNNW (SEQ ID NO:68), SNPWDSL LSVST (SEQ ID NO:69),

KTIEAHPPIYYAS (SEQ ID NO:70), EPDNWSLDFPRR (SEQ ID NO:71),
 HQHKPPPLTNNW (SEQ ID NO:72), GVVGKLGQRRTKKQRRQKK (SEQ ID
 NO:73), GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
 GRRTKKQRRQKKPP (SEQ ID NO:75).

5 47. The method of claim 45 wherein the cargo is glutathione.

 48. A kit for internalizing a GST-fusion protein into a cell
 comprising a peptide-cargo complex.

 49. The kit according to claim 48 wherein the peptide has the
 amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID
 10 NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3),
 PIRRRKKLRLRK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5),
 MHKRPTTPSRKM (SEQ ID NO:6), RQSRRRPLNIR (SEQ ID NO:7),
 RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9),
 QRIRKSKISRTL (SEQ ID NO:10), PSKRLLHNNLRR (SEQ ID NO:11),
 15 HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHSH (SEQ ID NO:13),
 PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15),
 GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17),
 NKRLIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25),
 AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),
 20 LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29),
 YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31),
 AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33),
 QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35),
 HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),
 25 FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),
 FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
 SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),

SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
 LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51),
 LPHPVLHMGPLR (SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53),
 5 NYTTYKSHFQDR (SEQ ID NO:54), AIPNNQLGFPEK (SEQ ID NO:55),
 NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT (SEQ ID NO:57),
 DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
 HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61),
 MTPSSLSTLPWP (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63),
 10 QPIIITSPYLPS (SEQ ID NO:64), TPKTMTQTYDFS (SEQ ID NO:65),
 NSGTMQSASRAT (SEQ ID NO:66), QAASRVENYMHK (SEQ ID NO:67),
 HQHKPPPLTNNW (SEQ ID NO:68), SNPWDSLLSVST (SEQ ID NO:69),
 KTIEAHPPYYAS (SEQ ID NO:70), EPDNWSLDFPRR (SEQ ID NO:71),
 HQHKPPPLTNNW (SEQ ID NO:72), GVVGKLGQRRTKKQRRQKK (SEQ ID
 15 NO:73), GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
 GRRTKKQRRQKKPP (SEQ ID NO:75).

50. The kit according to claim 48 wherein the cargo is glutathione.

51. An immunogen comprising a peptide-cargo complex wherein
 said peptide has an amino acid sequence selected from the group consisting of
 20 KRIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2),
 PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4),
 RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
 RQSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
 SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10),
 25 PSKRLHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12),
 PQNRLQIRRHKS (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14),
 SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
 SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
 HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26),

- PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28),
 SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30),
 SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
 SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
 5 HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
 LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38),
 FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40),
 FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42),
 SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44),
 10 SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46),
 SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48),
 SSSIFPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50),
 LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52),
 VSKQPYMWNNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
 15 AIPNNQLGFPFK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56),
 YPYDANHTRSPT (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58),
 TLPSPLALLTVH (SEQ ID NO:59), HPGSPFPPEHRP (SEQ ID NO:60),
 TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP (SEQ ID NO:62),
 VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
 20 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
 QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
 SNPWDSLLSVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
 EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
 GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
 25 GRRTKKQRRQKKPPRYMILGLLAALAAVCSAA (SEQ ID NO:74) and
 GRRTKKQRRQKKPP (SEQ ID NO:75).

52. The immunogen of claim 51 wherein the peptide has an amino acid sequence RRQRRTSKLMKR (SEQ ID NO:5).

53. The immunogen of claim 51 wherein the peptide has an amino acid sequence GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73).

54. The immunogen of claim 51 wherein the cargo is selected from the group consisting of a polynucleotide, a polypeptide, a protein, a virus, a modified
5 virus, a viral vector, and a plasmid.

55. The immunogen of claim 51 wherein the cargo is an antigen.

56. The immunogen of claim 51 wherein the cargo is an HIV protein selected from the group consisting of Gag, Pol, Env, Tat, Nef, Vpr, Vpv, Rev.

10 57. A method for eliciting an immune response in a subject comprising administering to a target cell of said subject an immunogen comprising a peptide-cargo complex wherein said peptide has an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
15 RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHKS (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14),
20 SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRLIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30),
25 SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38),

- FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40),
 FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42),
 SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44),
 SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46),
 5 SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48),
 SSSIIPPWLSFF (SEQ ID NO:49), LNVPPSWFLSQR (SEQ ID NO:50),
 LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR (SEQ ID NO:52),
 VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
 AIPNNQLGFPPK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56),
 10 YPYDANHTRSPT (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58),
 TLPSPLALLTVH (SEQ ID NO:59), HPGSPFPPEHRP (SEQ ID NO:60),
 TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP (SEQ ID NO:62),
 VLGQSGYLMPMR (SEQ ID NO:63), QPIIITSPYLPS (SEQ ID NO:64),
 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
 15 QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
 SNPWDSLLSVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
 EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
 GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
 GRRTKKQRRQKKPPRYMILGLLAALAAVCSAA (SEQ ID NO:74) and
 20 GRRTKKQRRQKKPP (SEQ ID NO:75)..

58. The method of claim 57 wherein the target cell is a mucosal cell.

59. The method of claim 58 wherein the mucosal cell is a cervical mucosal cell.

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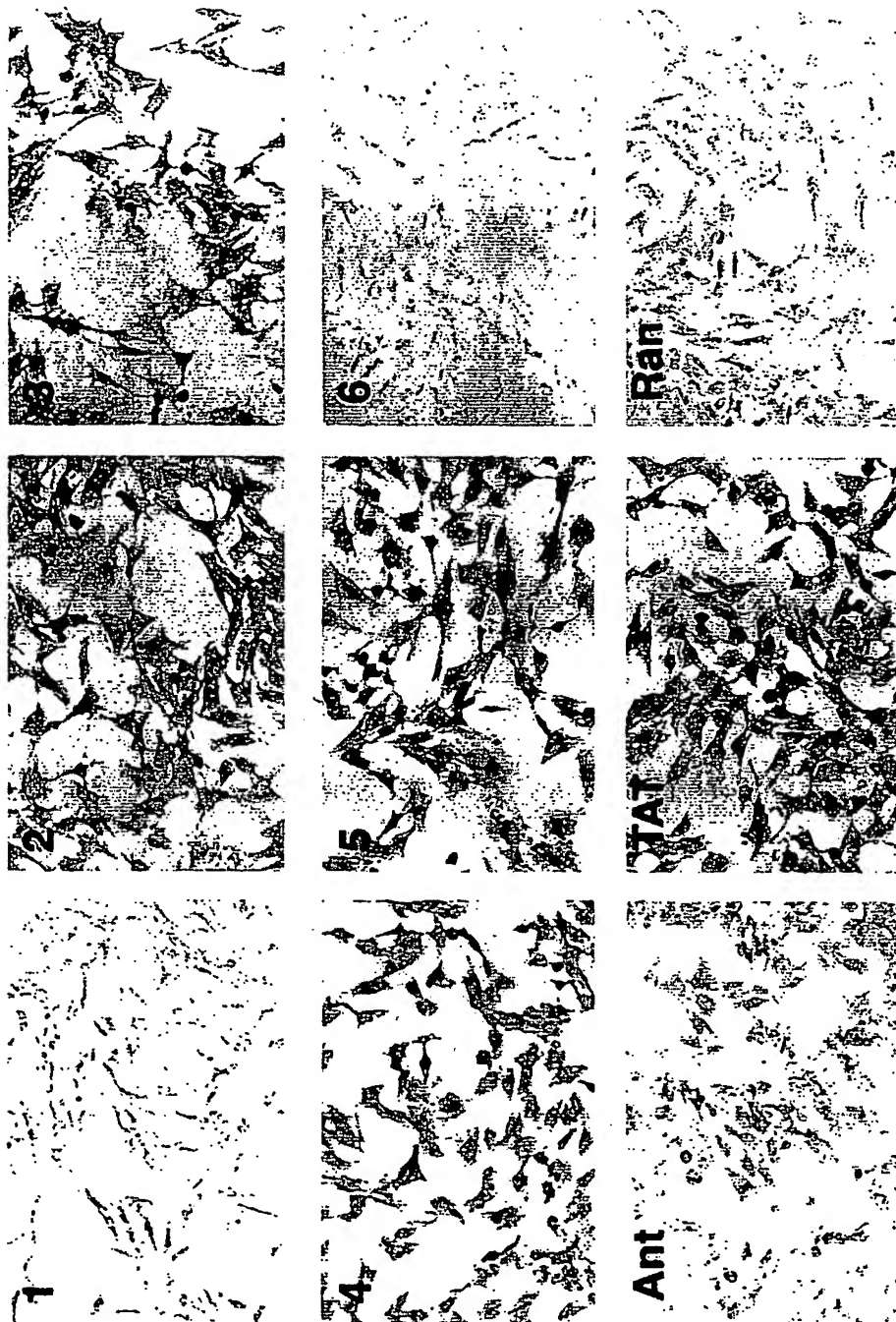


FIG. 1A

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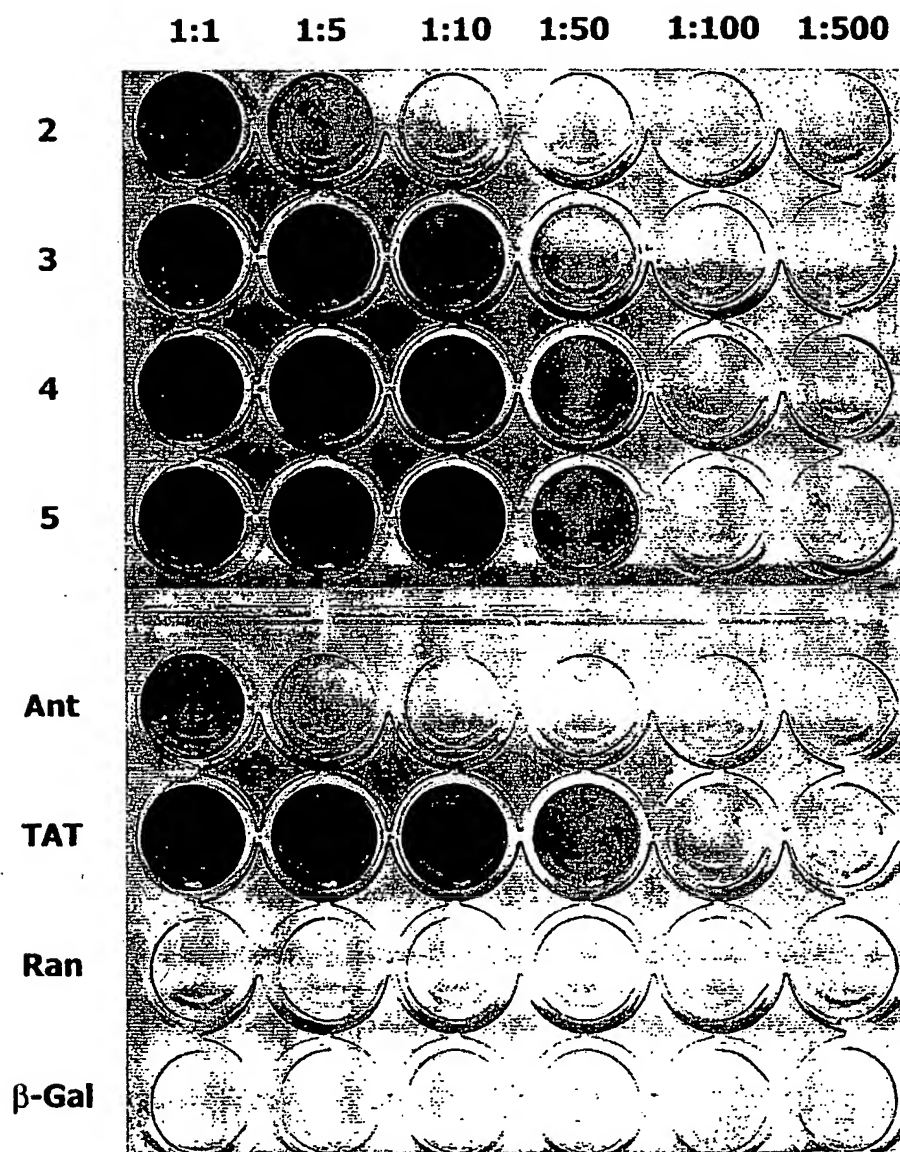


FIG.1B

FIG.2B



FIG.2D

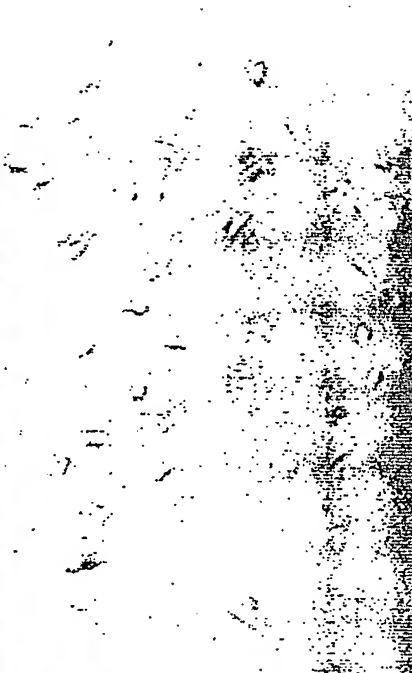


FIG.2A



FIG.2C

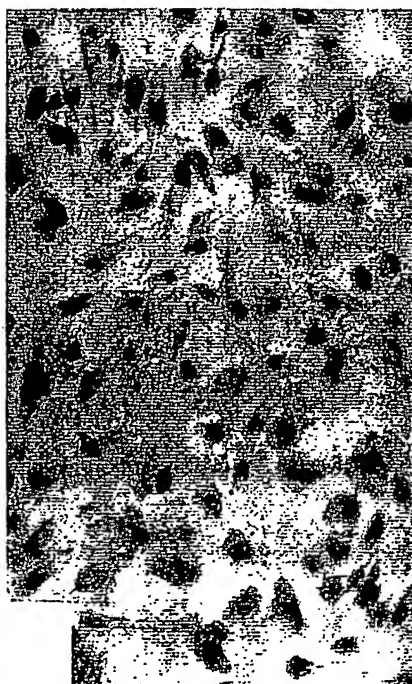


FIG.3B

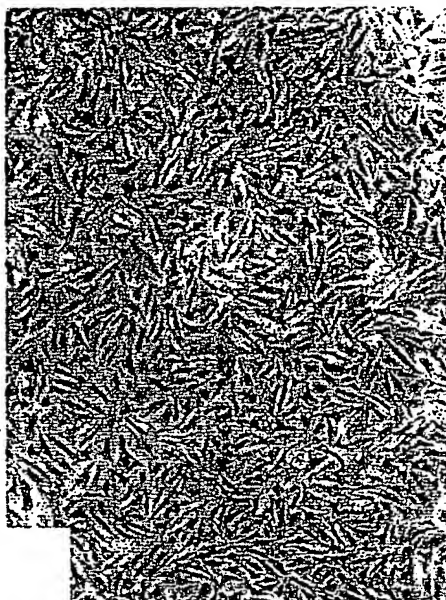


FIG.3D

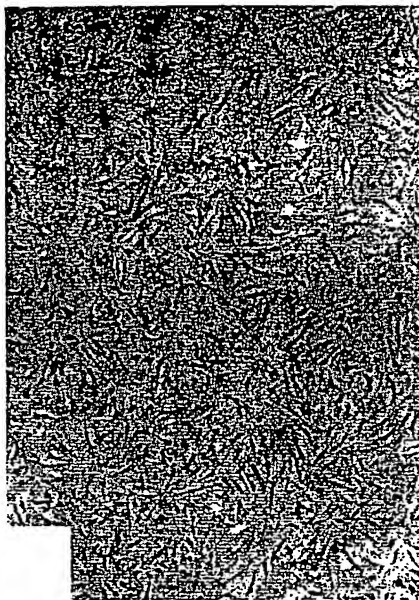
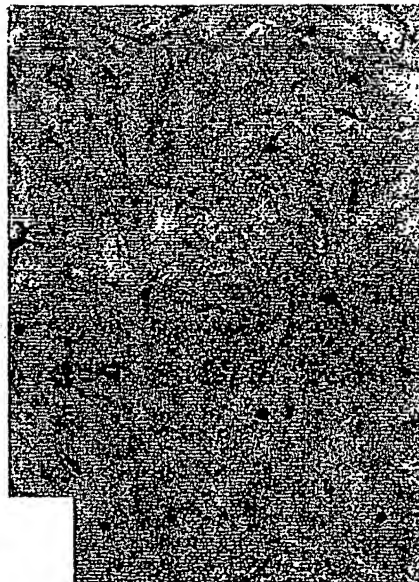


FIG.3A



FIG.3C



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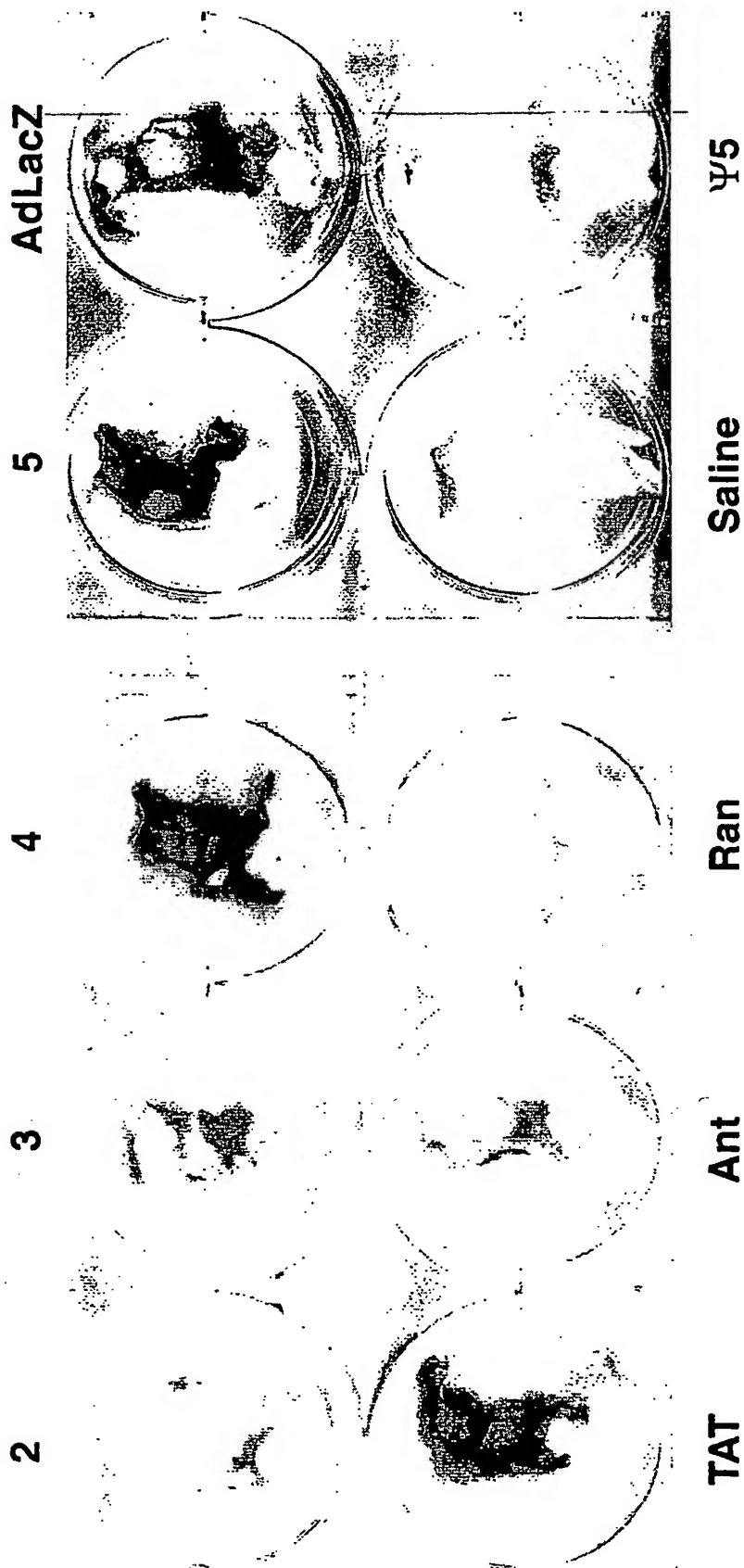


FIG.4A



FIG. 4B

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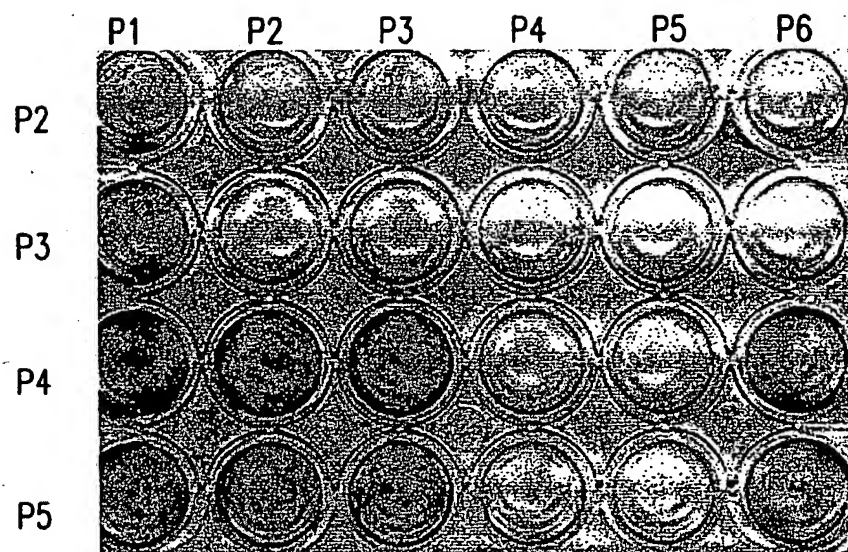


FIG.5

FIG. 6C



FIG. 6B



FIG. 6A



FIG. 6F



FIG. 6E

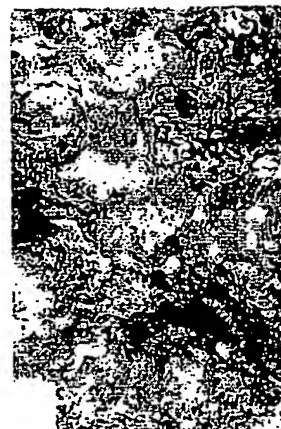
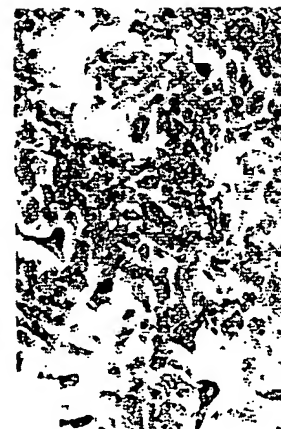


FIG. 6D



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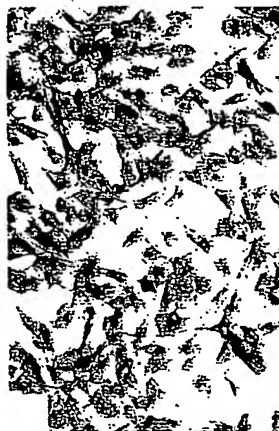


FIG. 6I

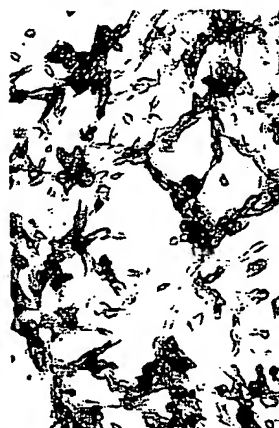


FIG. 6H



FIG. 6G

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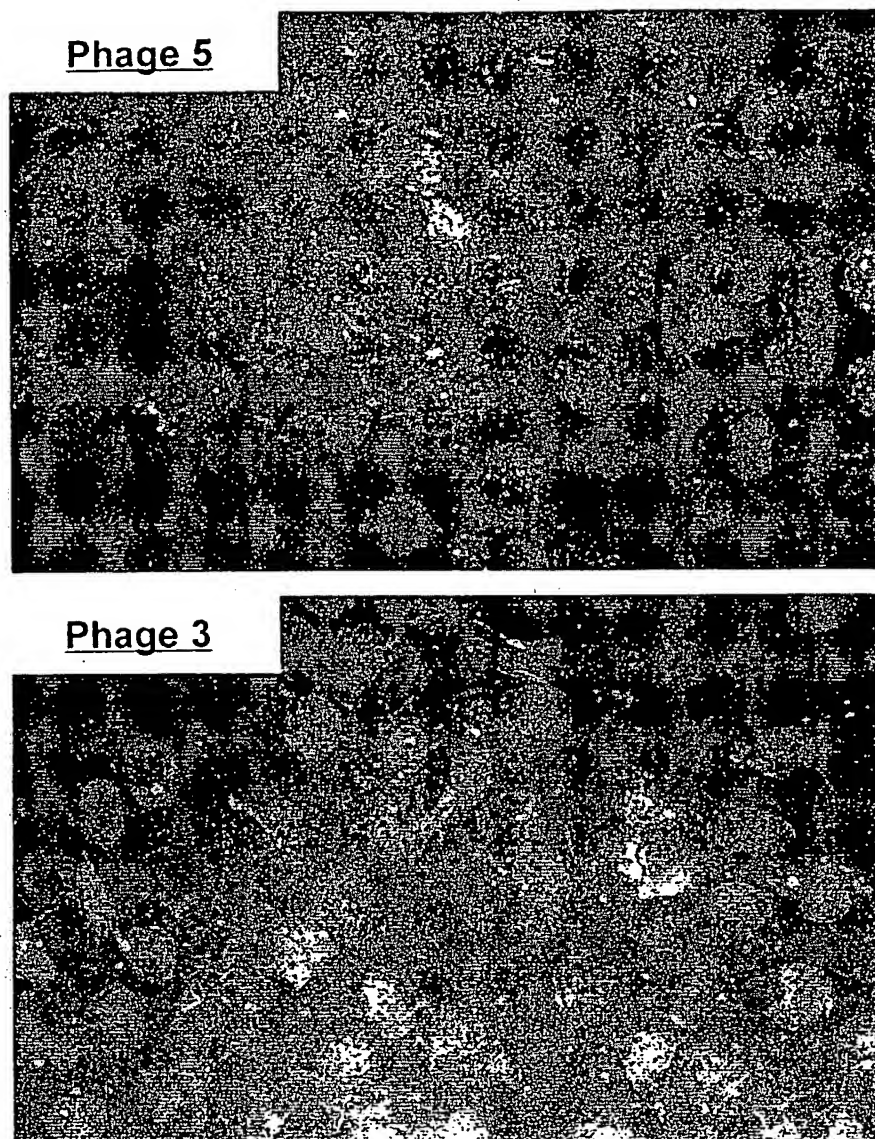


FIG. 7

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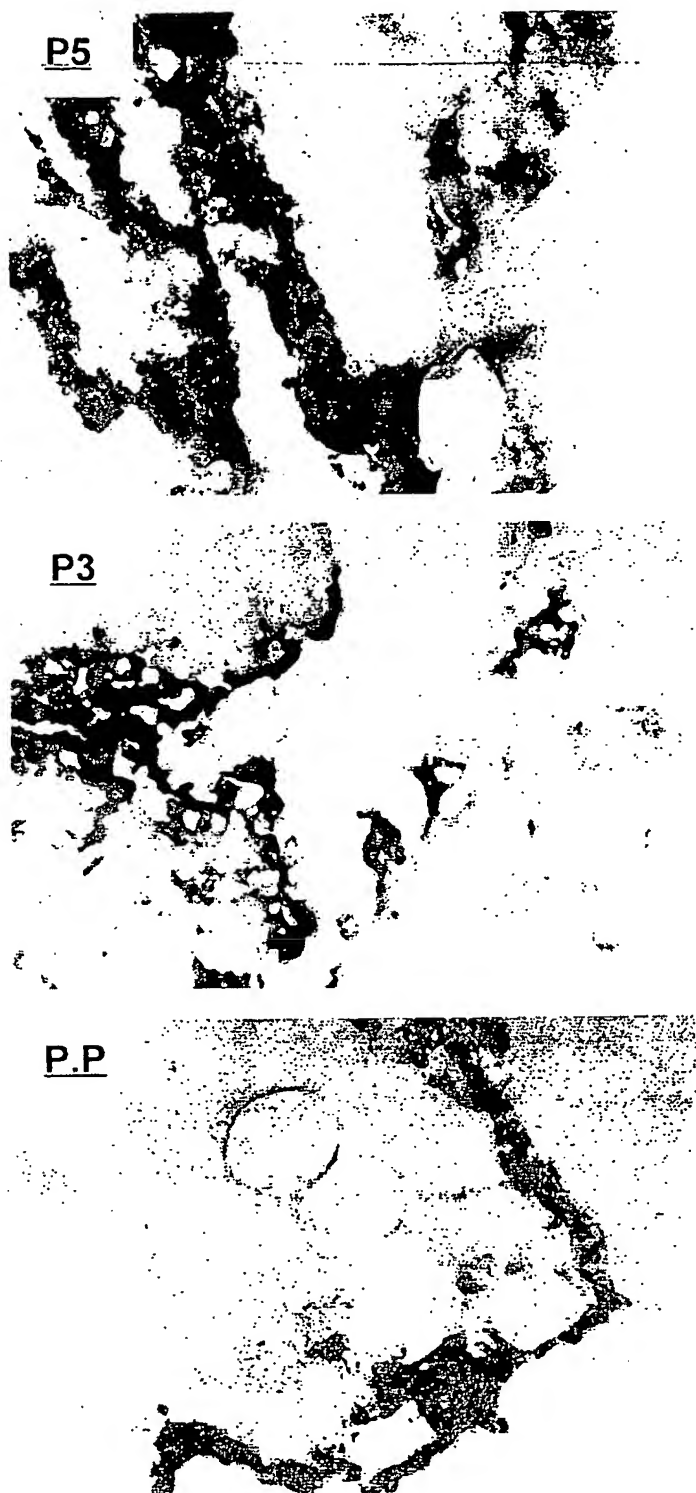


FIG.8

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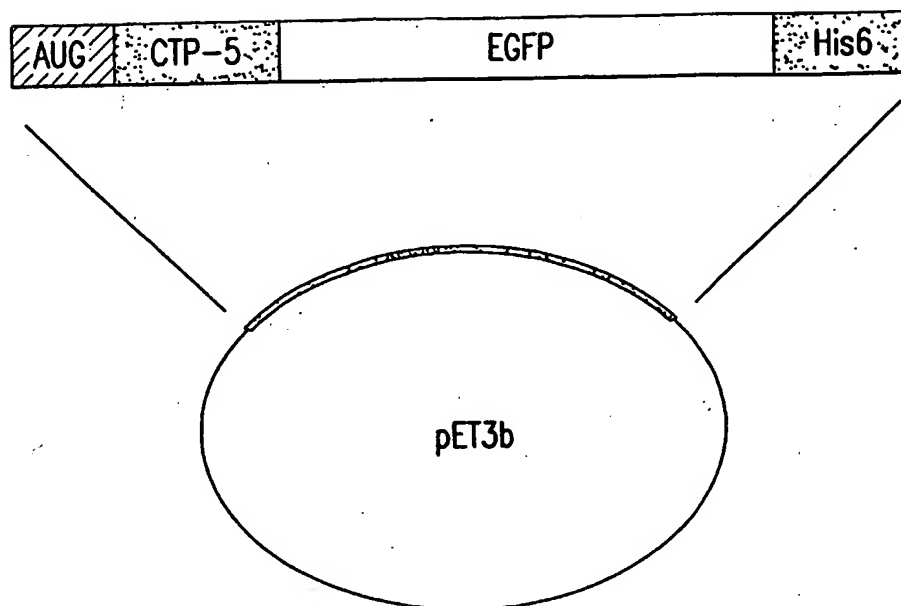


FIG.9A

FIG. 9F



FIG. 9G

FIG. 9D

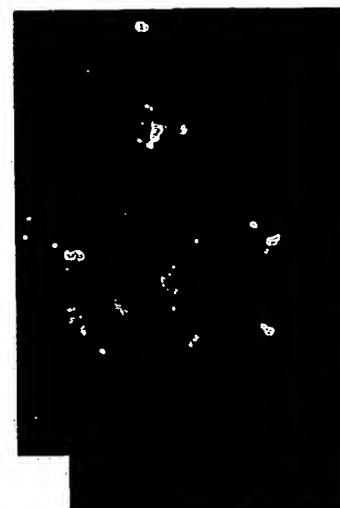


FIG. 9E

FIG. 9B



FIG. 9C

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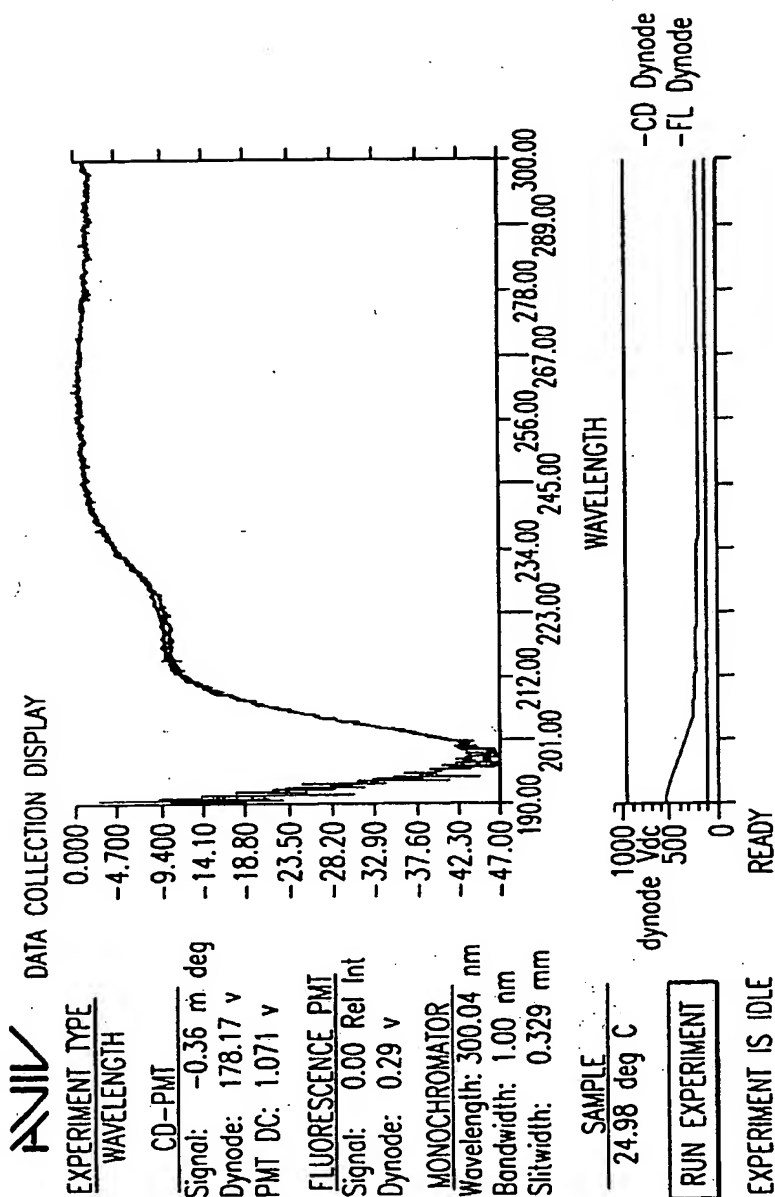


FIG.10A

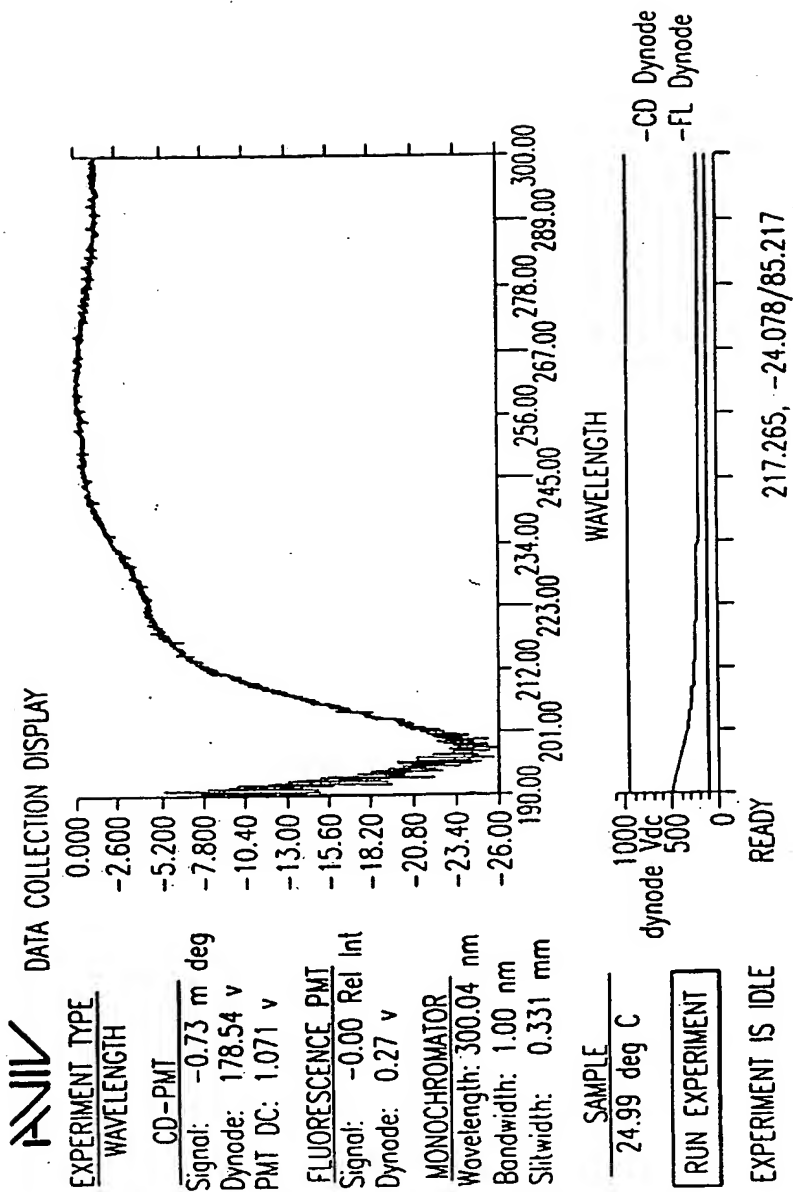


FIG.10B

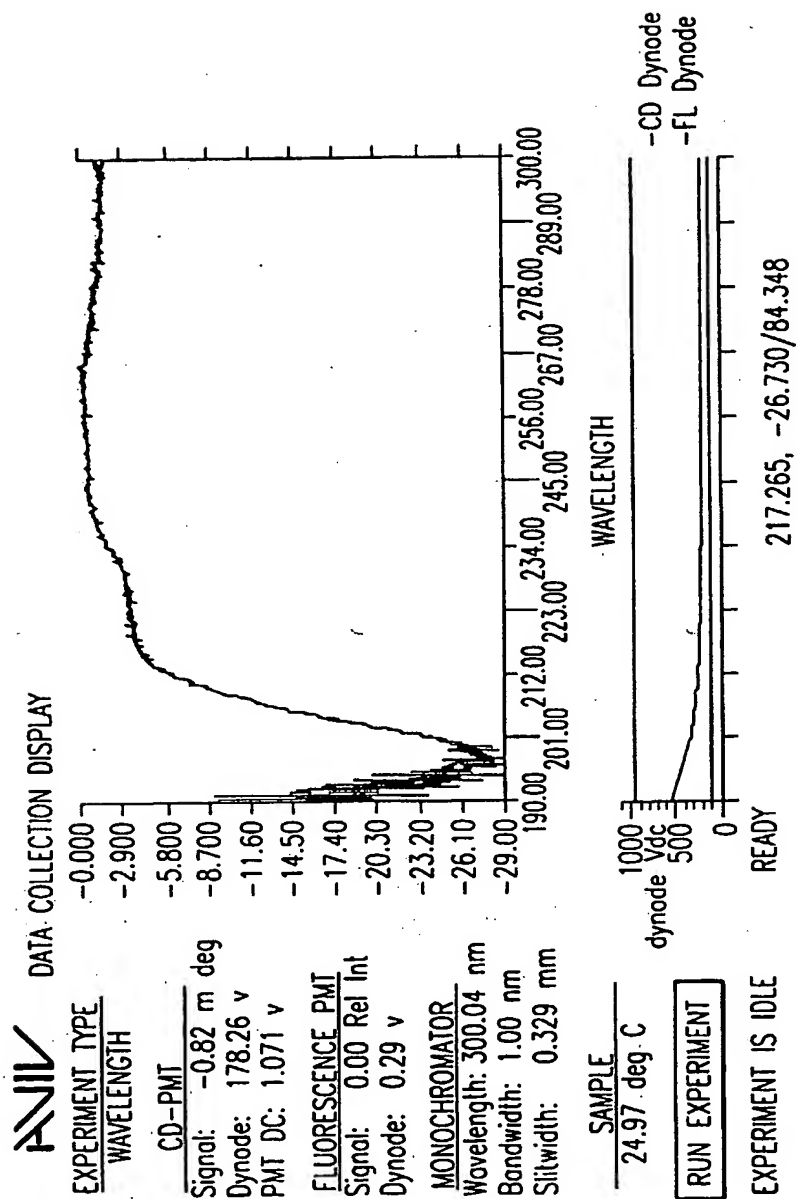


FIG.10C

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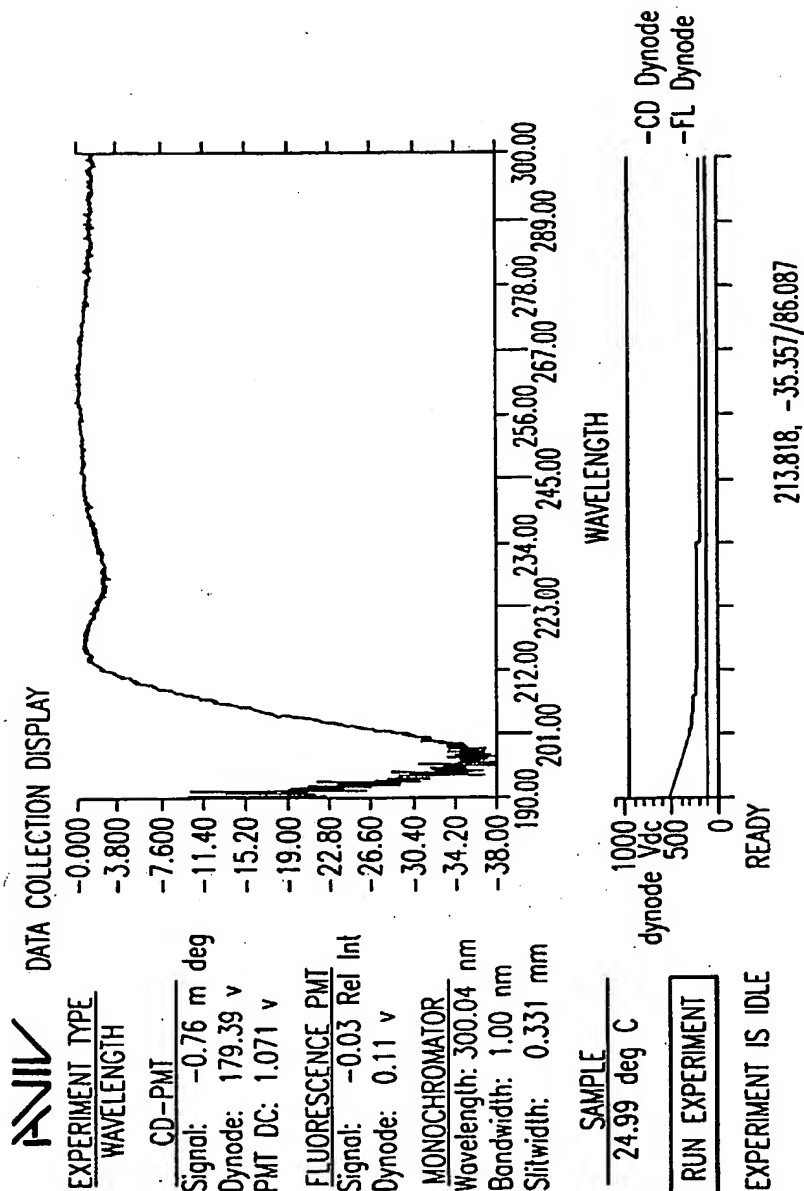


FIG.10D

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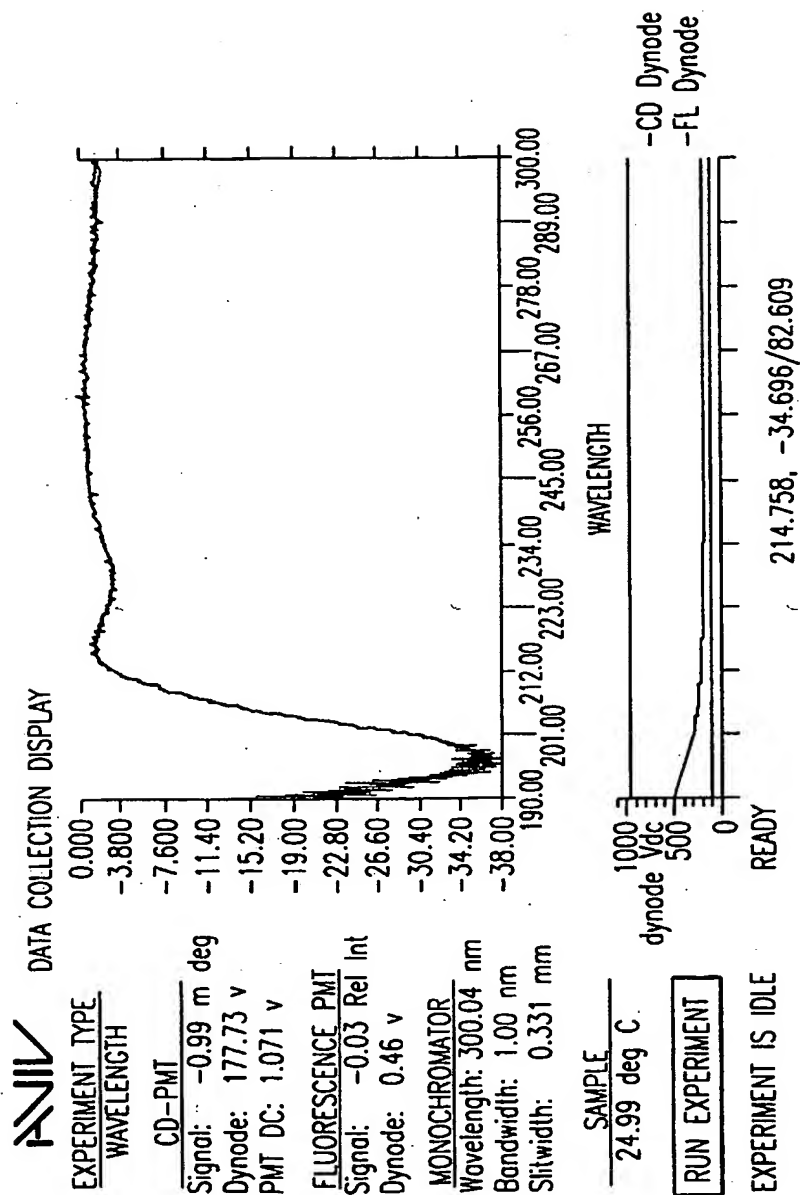


FIG.10E

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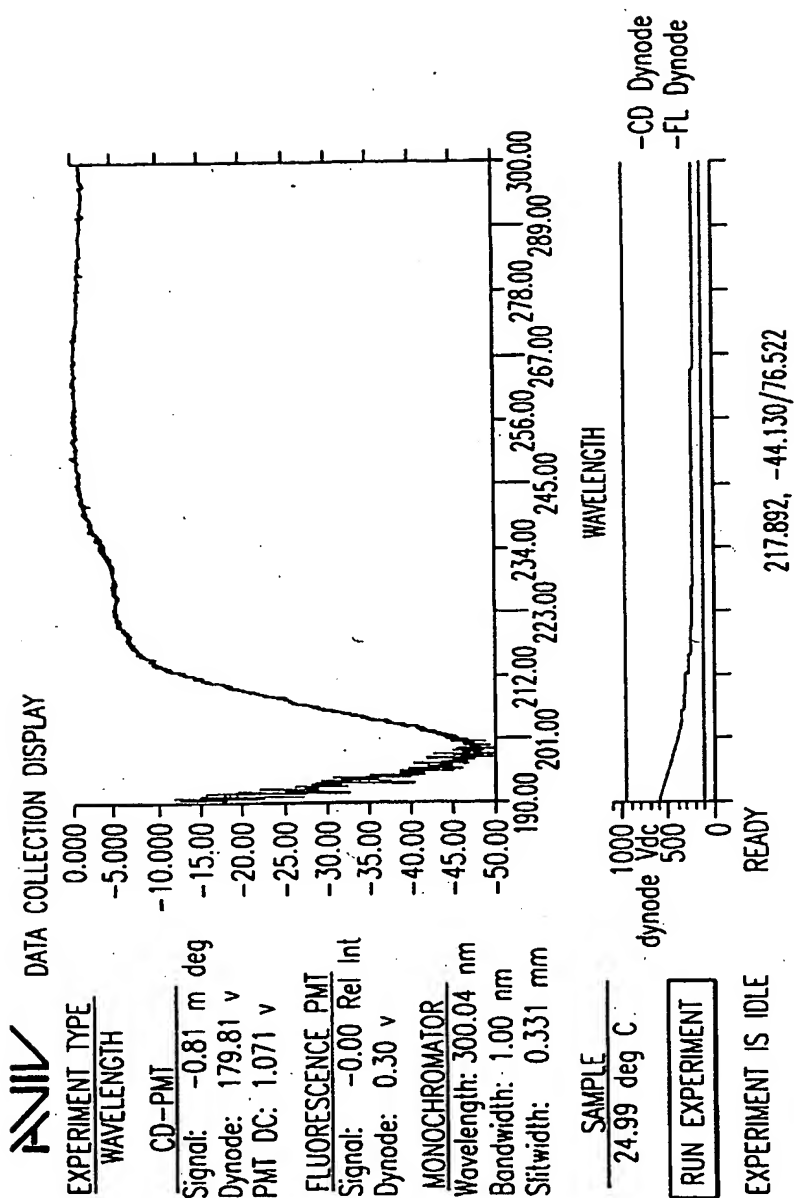


FIG.10F

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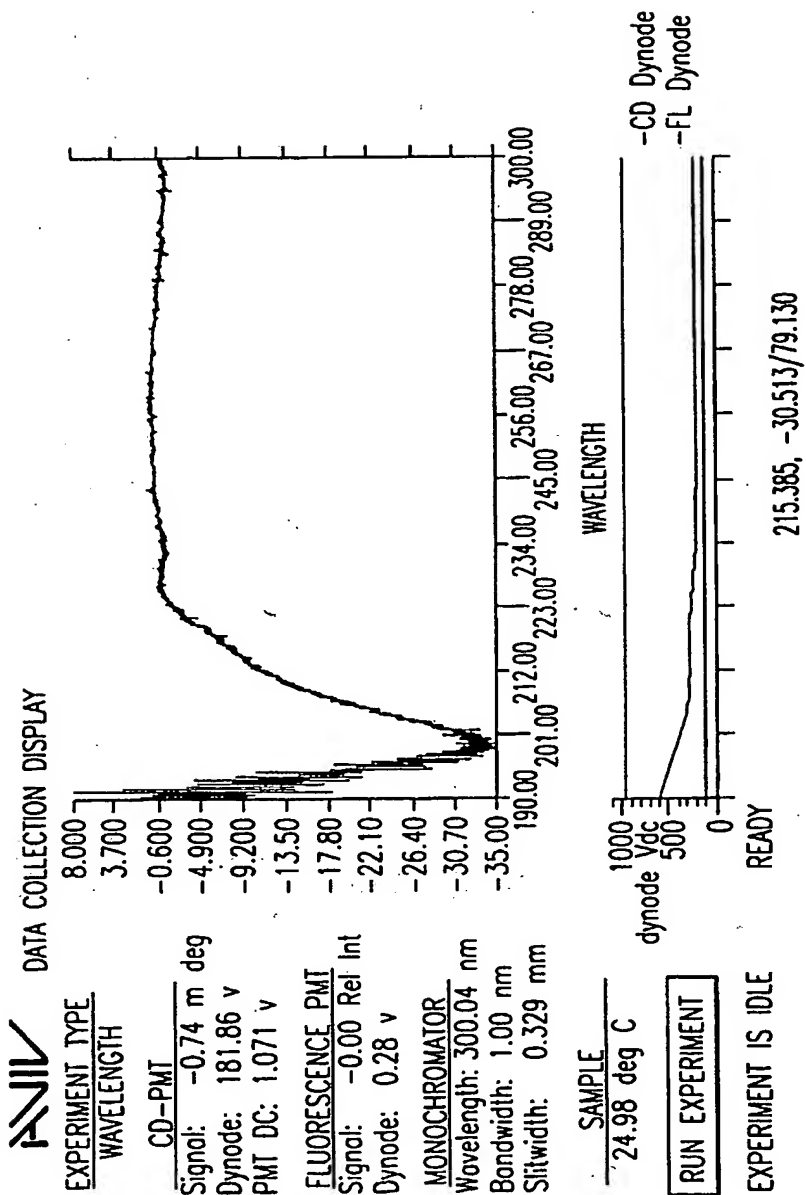


FIG.10G

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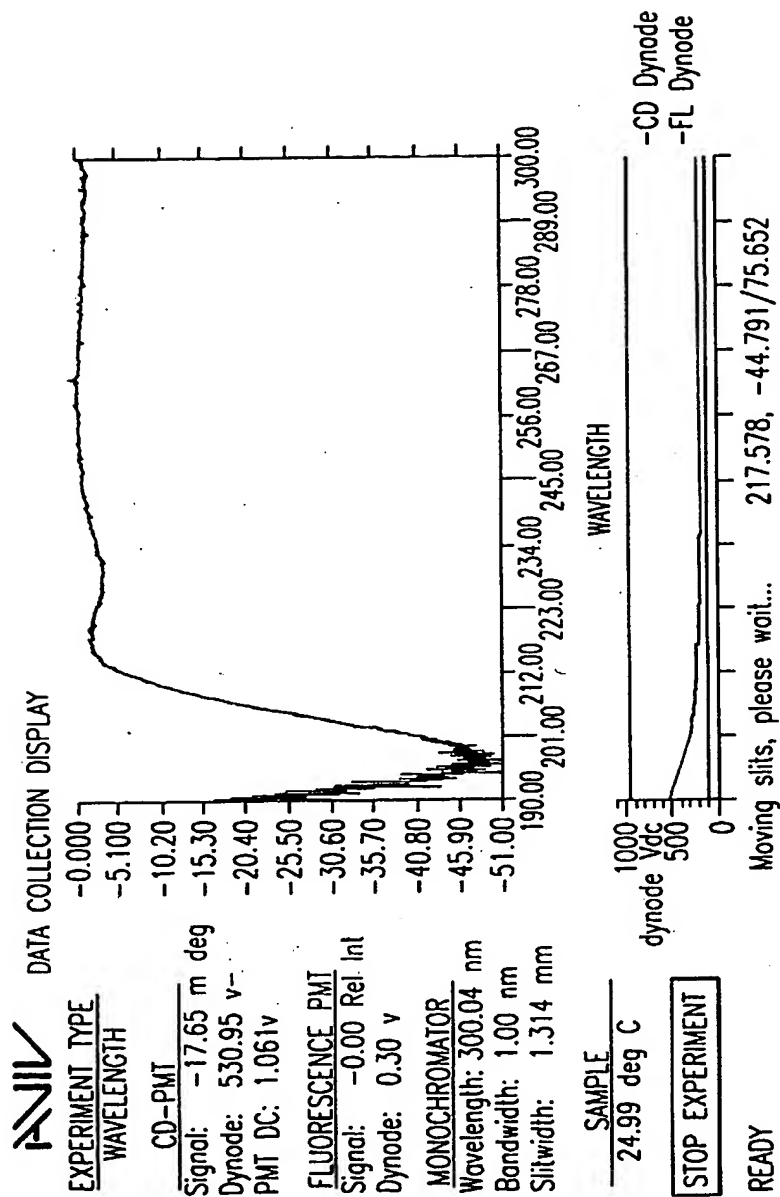


FIG.10H

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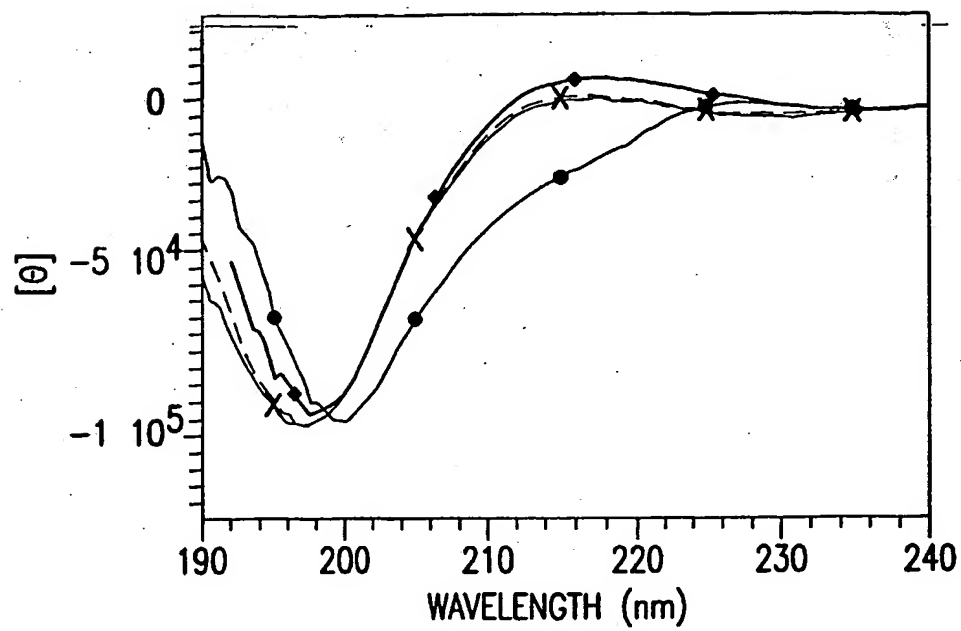


FIG. 11A

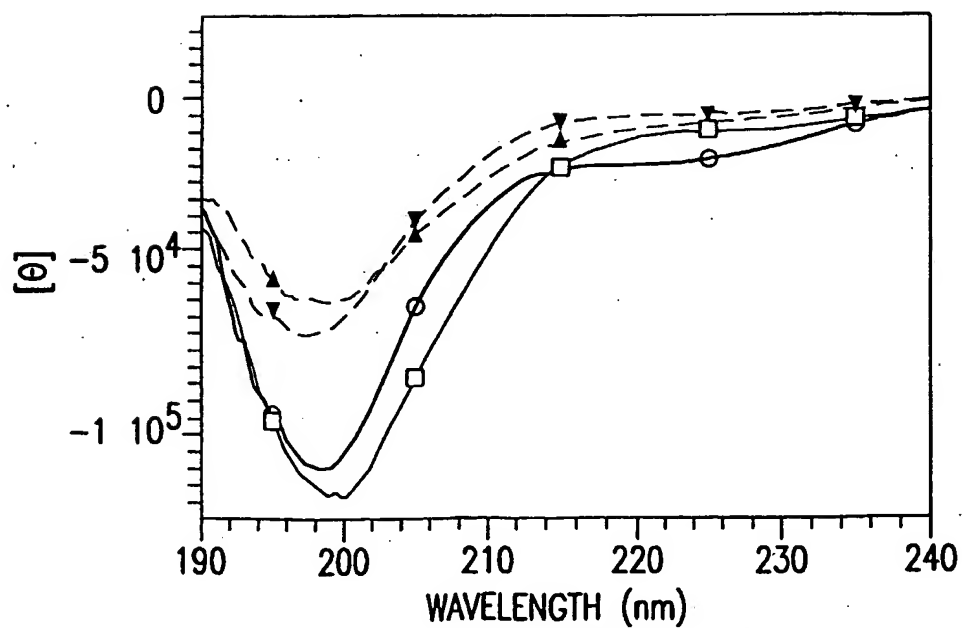


FIG. 11B

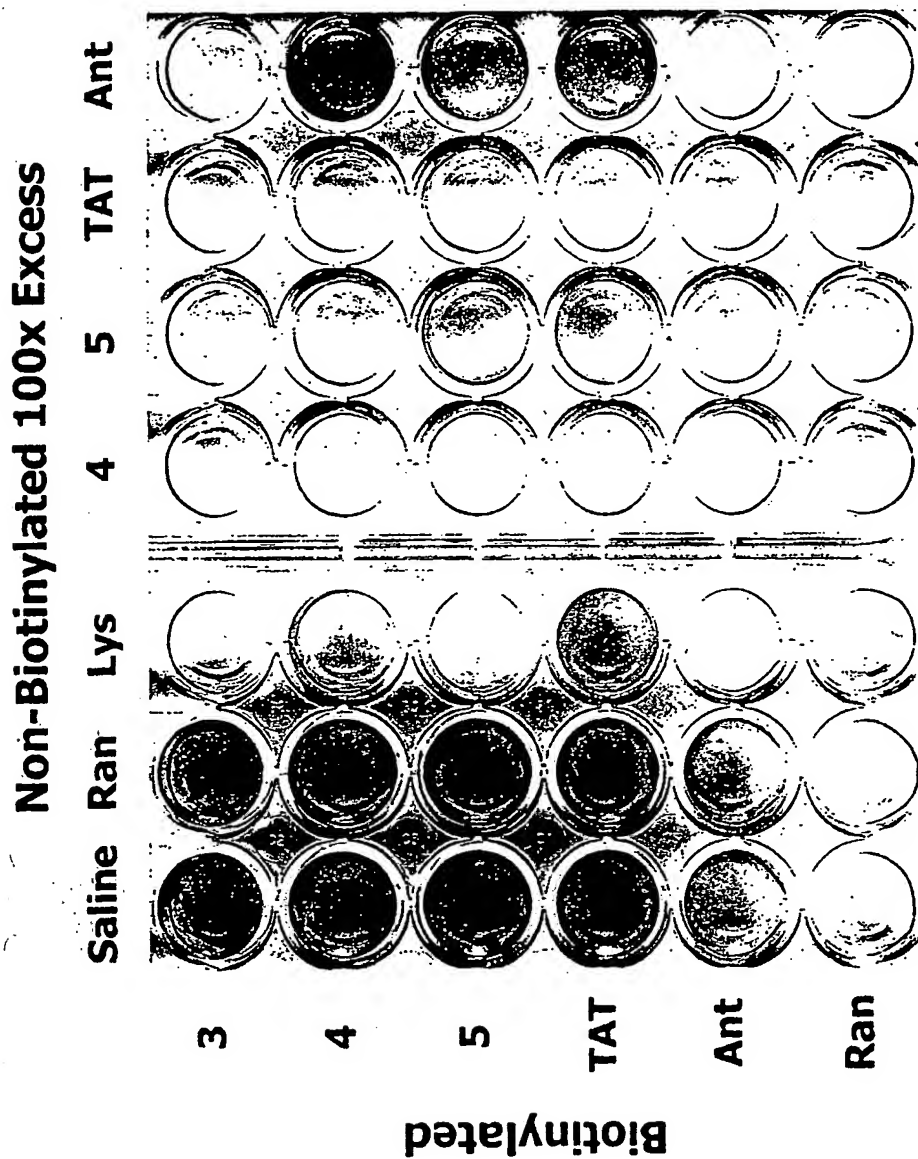


FIG.12

FIG. 13B



FIG. 13D



FIG. 13A



FIG. 13C



DP1 Impairs HIG-82 Viability.

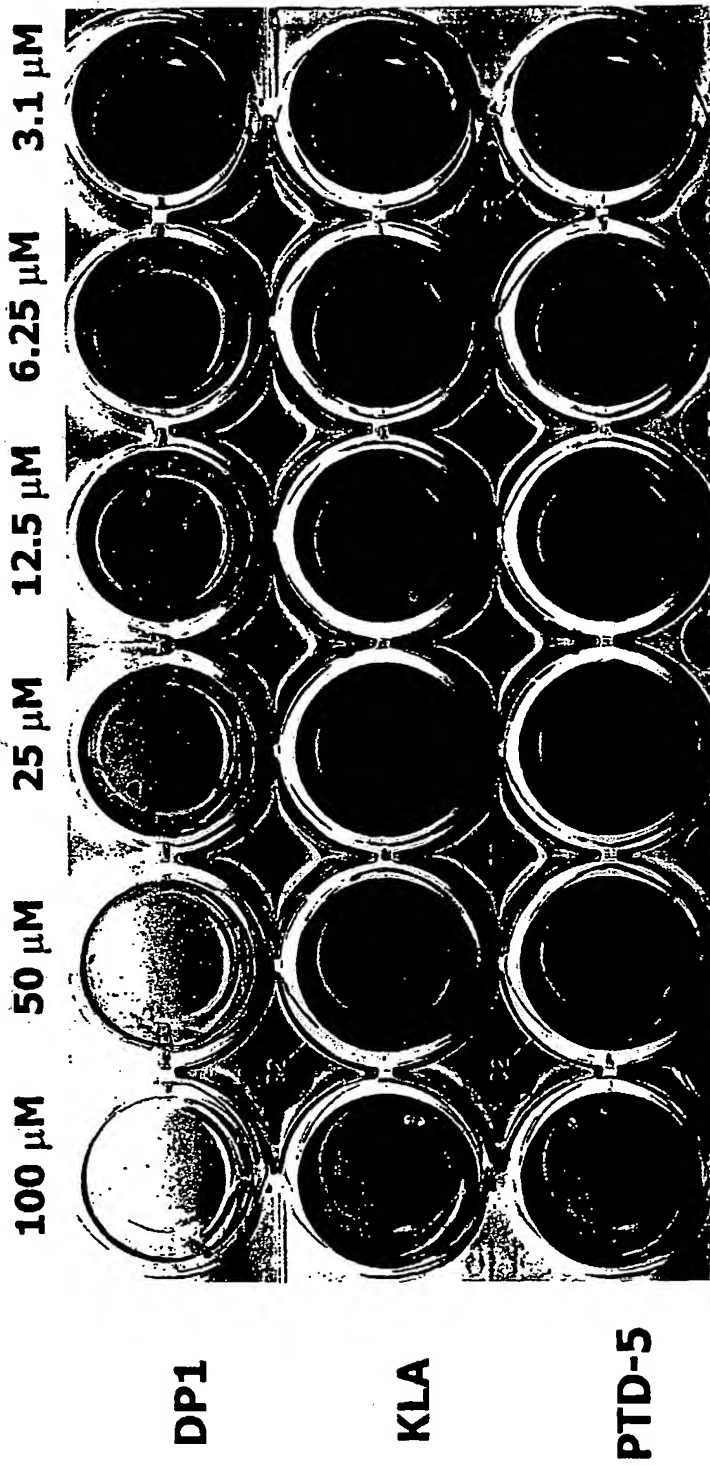


FIG.14

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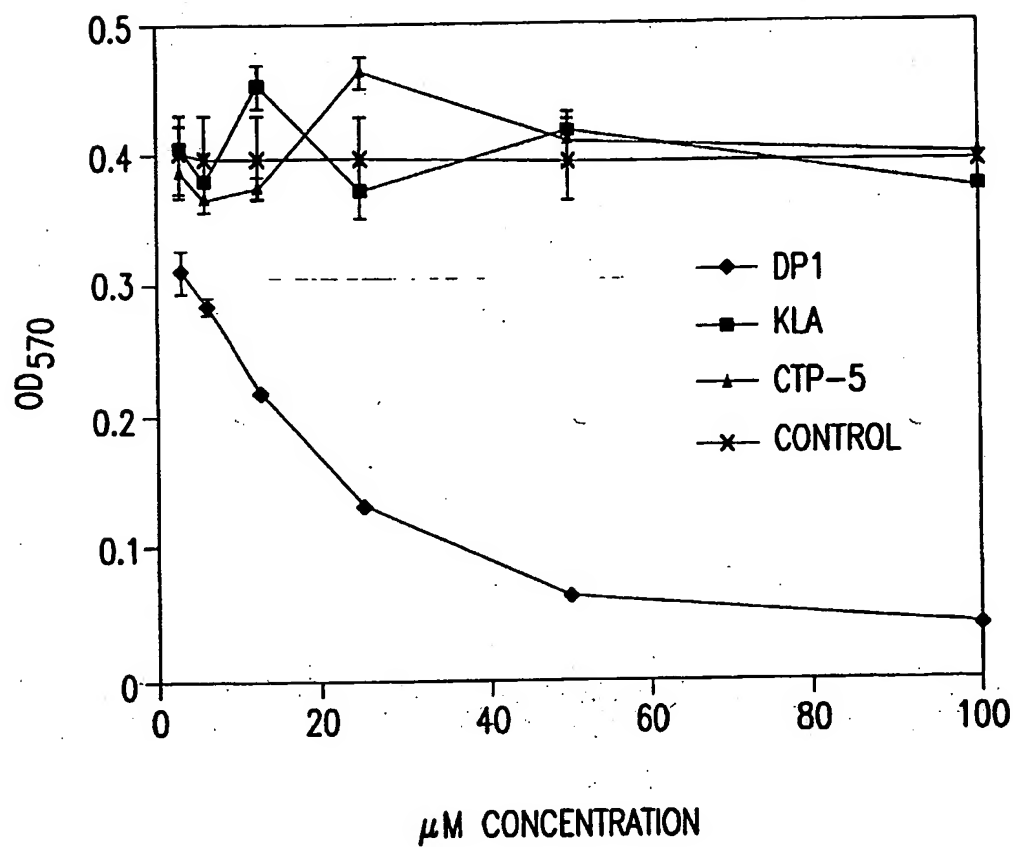
CTP-5-(KLAKLAK)₂ PEPTIDE IMPAIRS CELL
VIABILITY IN Hg 82 CELLS

FIG.15

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EFFECT OF CTP-5-(KLAKLAK)₂ PEPTIDE
ADMINISTRATION ON DAY 7 MCA205 TUMORS

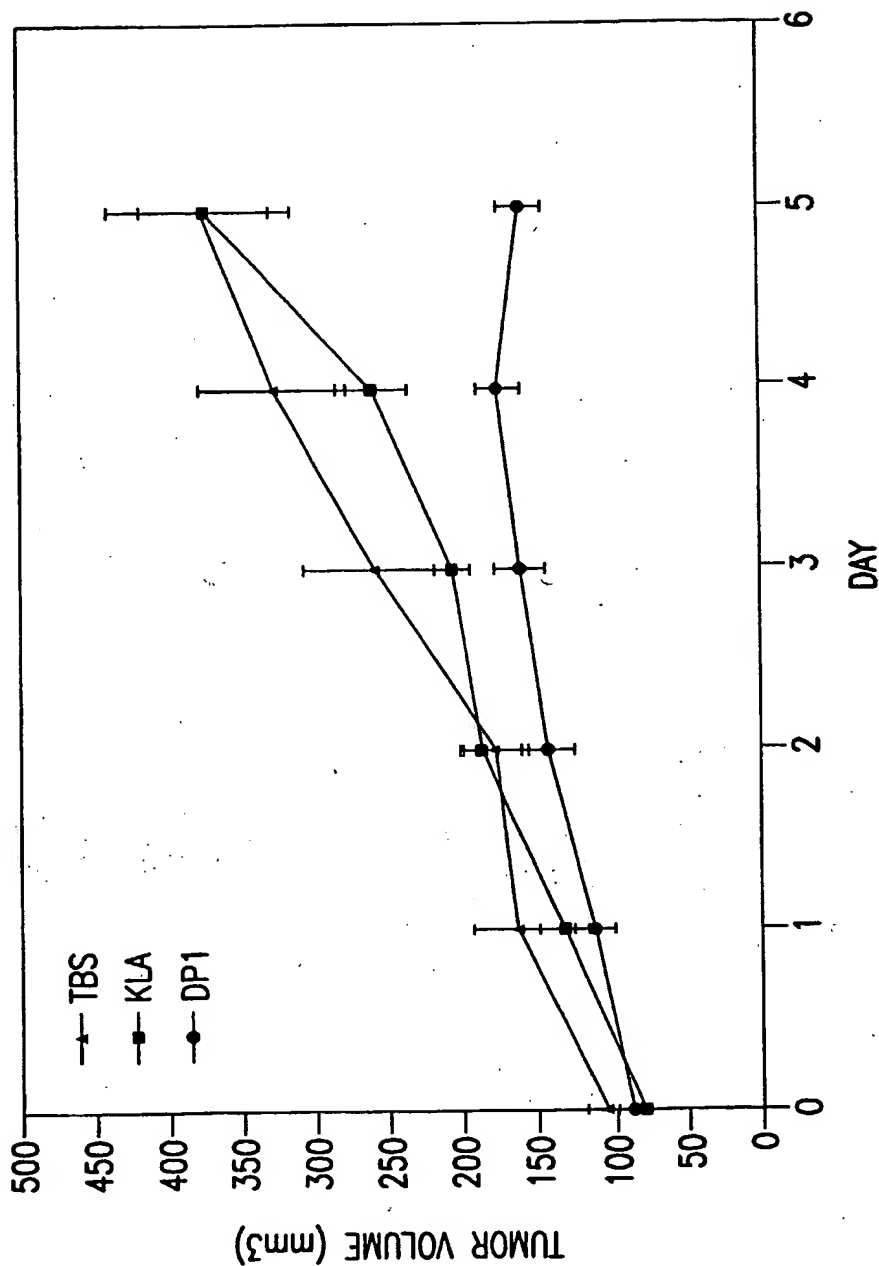


FIG.16A

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KLA



DP1

FIG. 16B

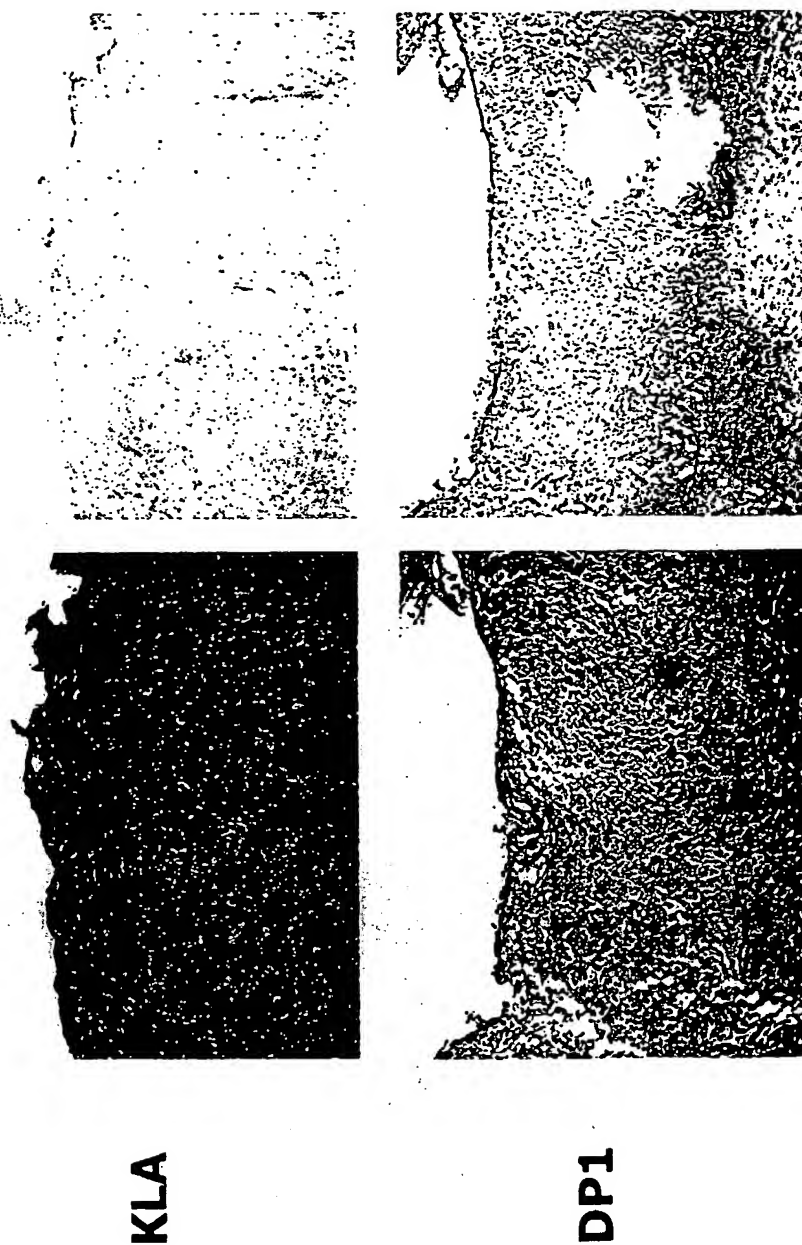


FIG. 16C

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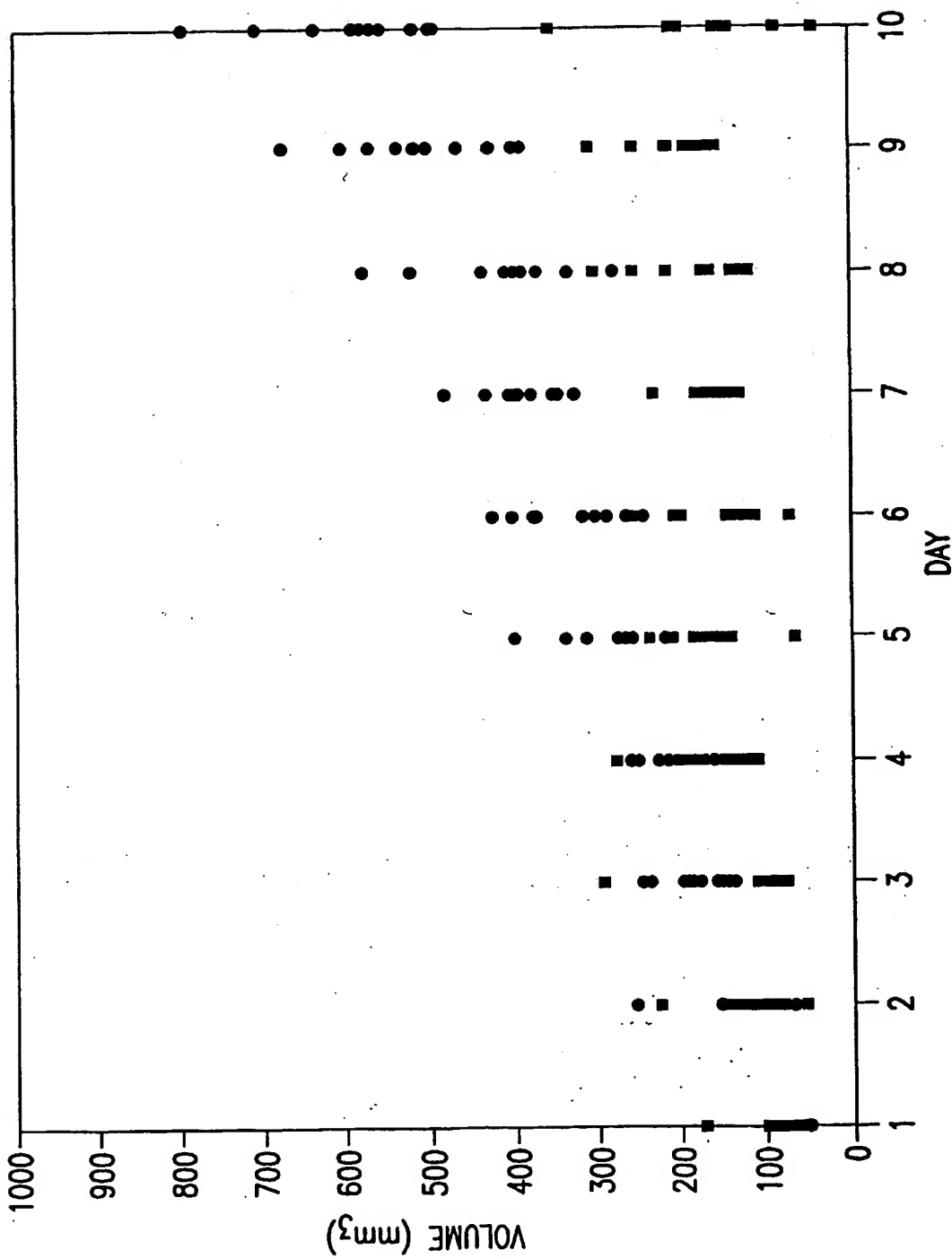


FIG. 16D

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**CD34⁺/LIN⁻ Stem Cells Are Transduced by a
CTP-5-Biotin/Avidin- β -Galactosidase
Complex**

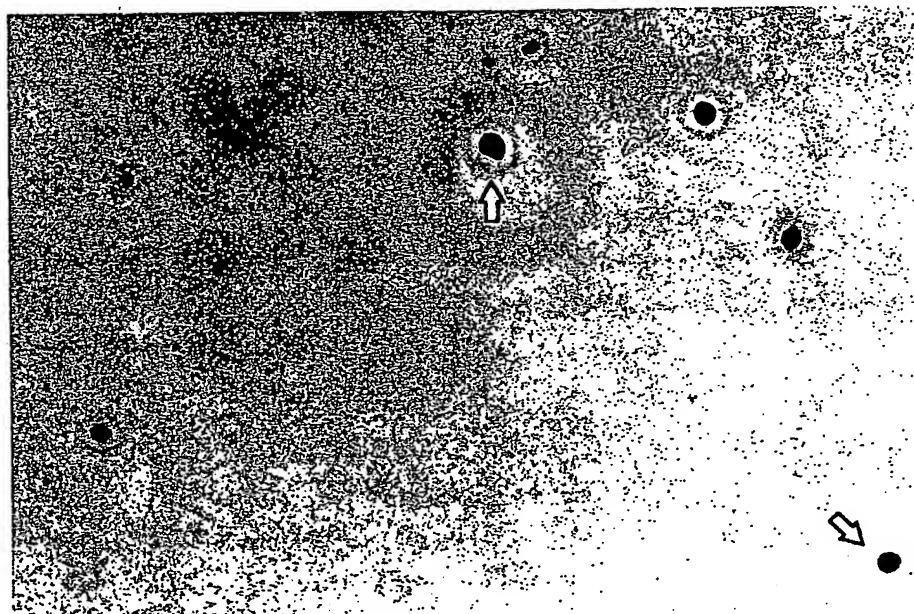


FIG.17

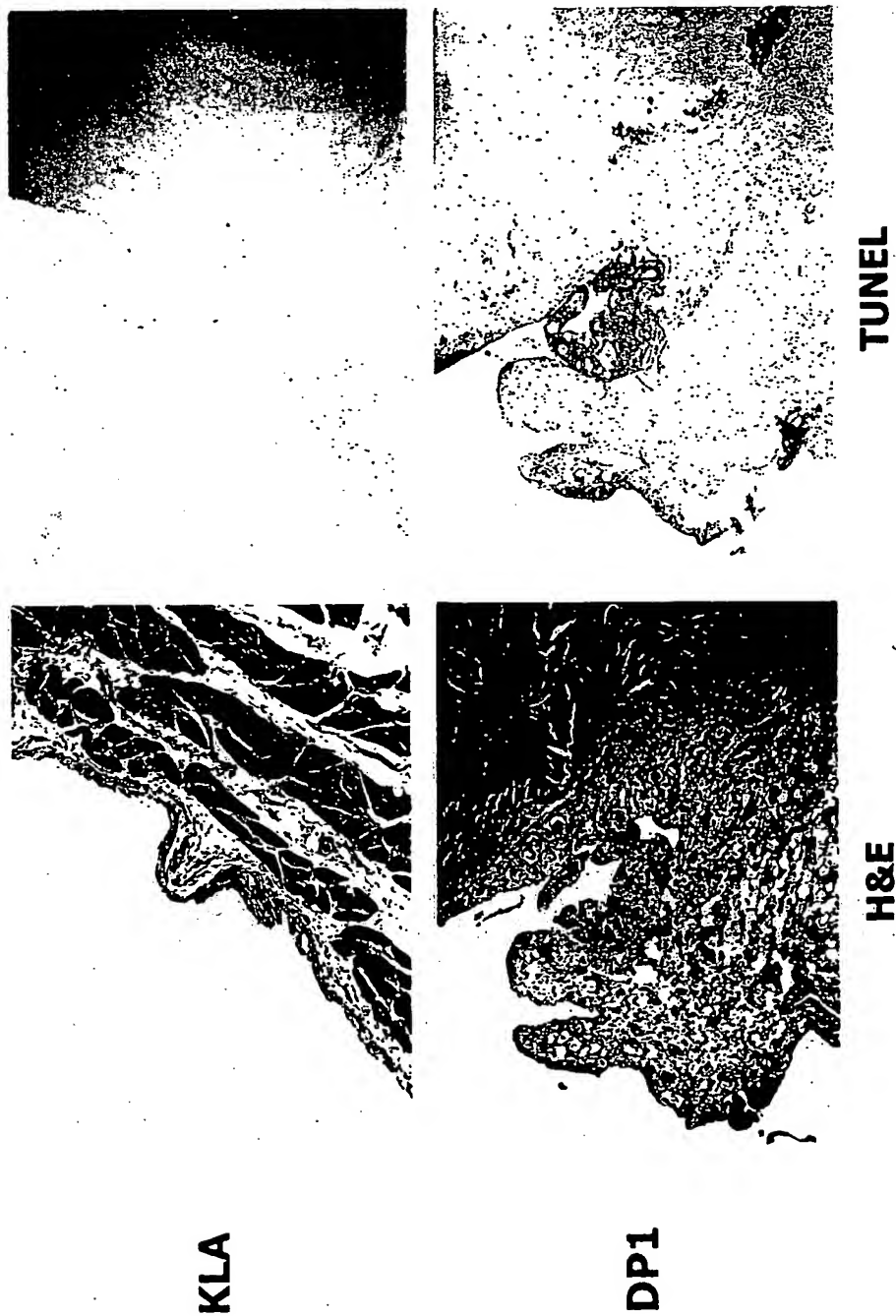


FIG.18

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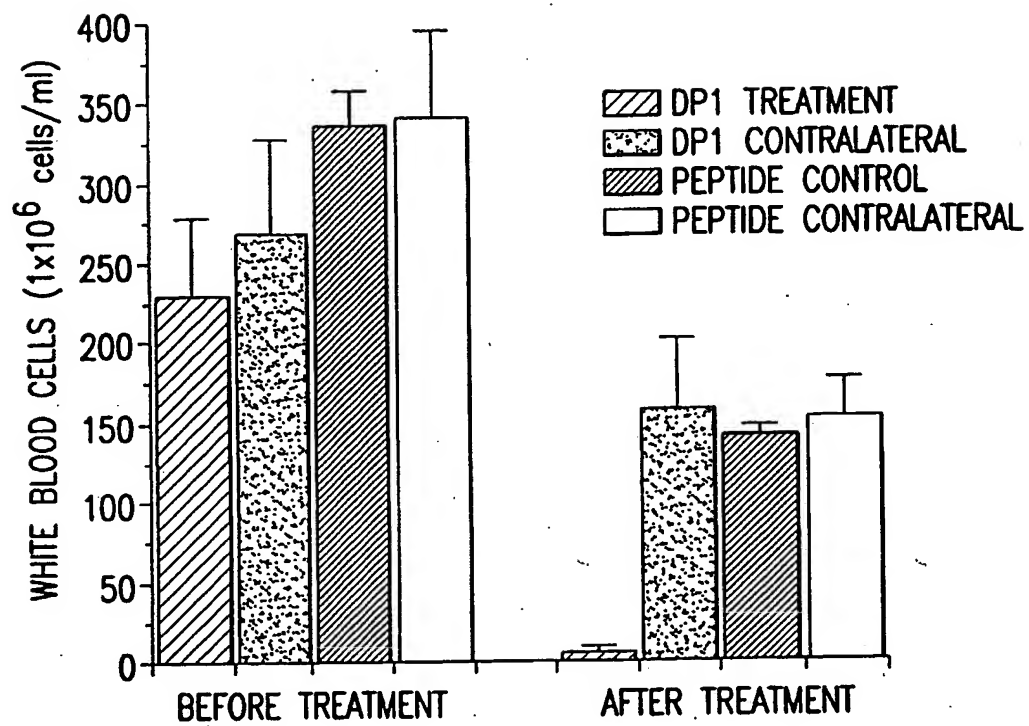


FIG.19

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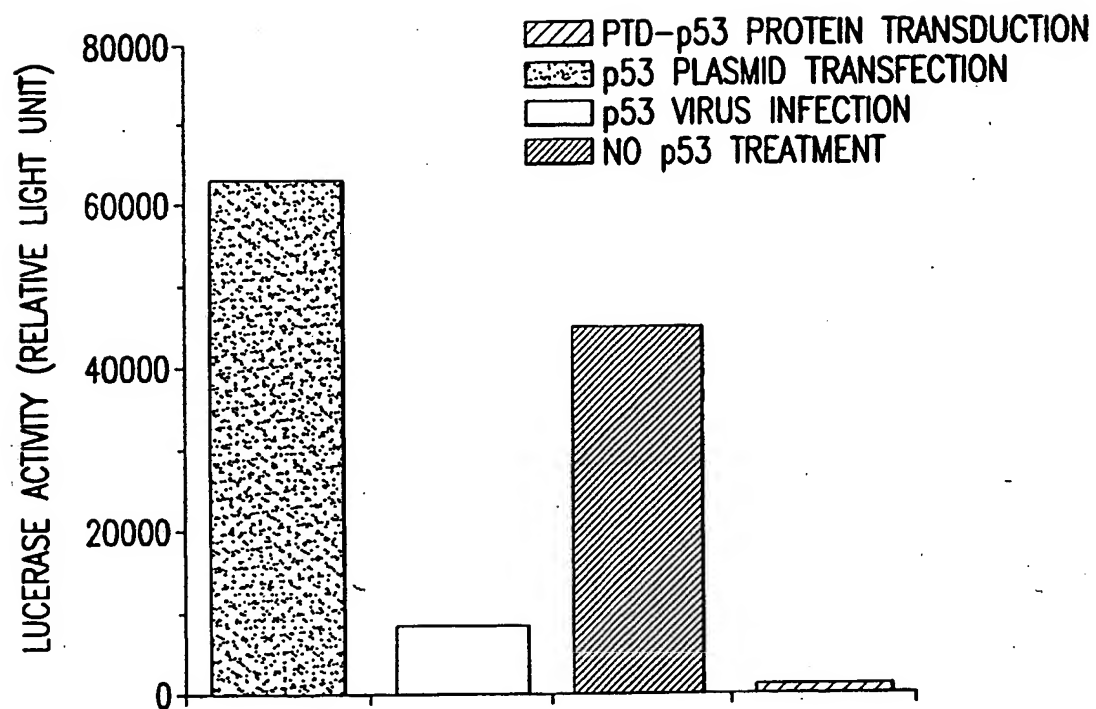


FIG.20

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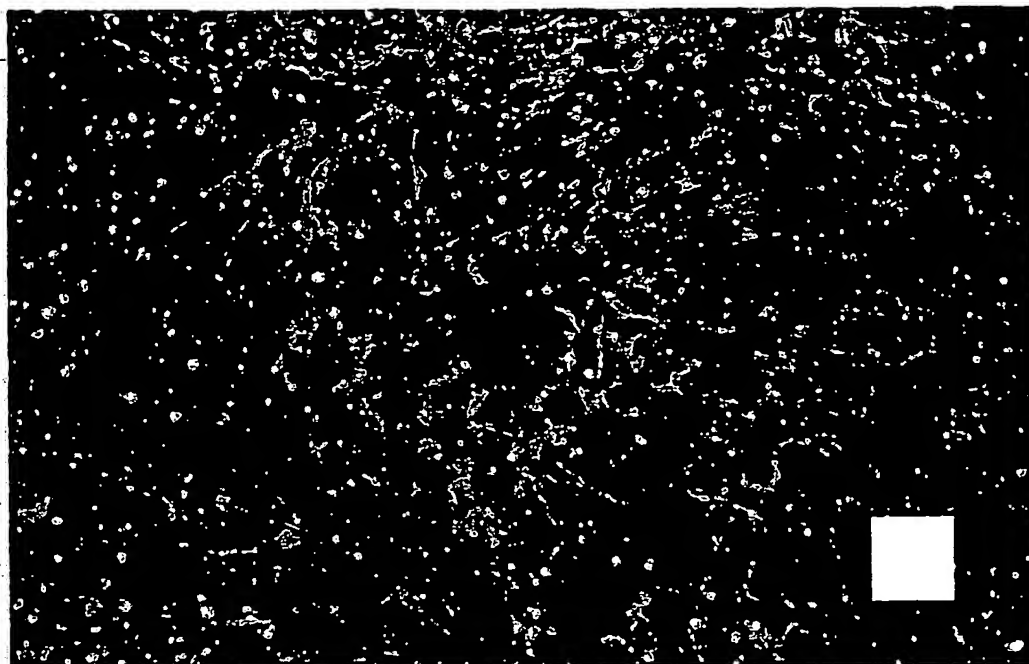


FIG.21A



FIG.21B

SUBSTITUTE SHEET (RULE 26)

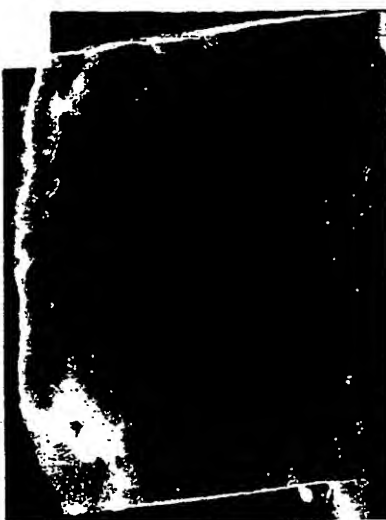


FIG. 22C



FIG. 22B



FIG. 22A

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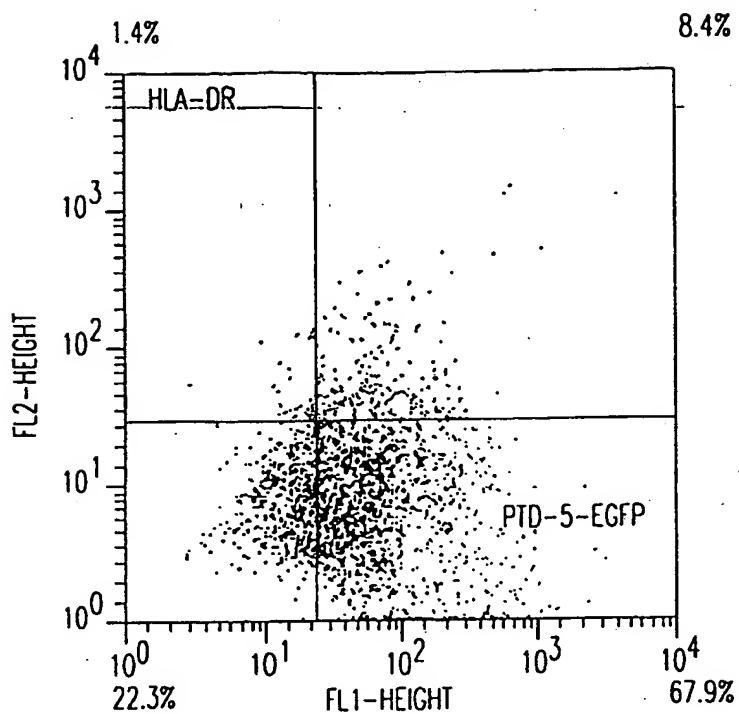


FIG. 22D

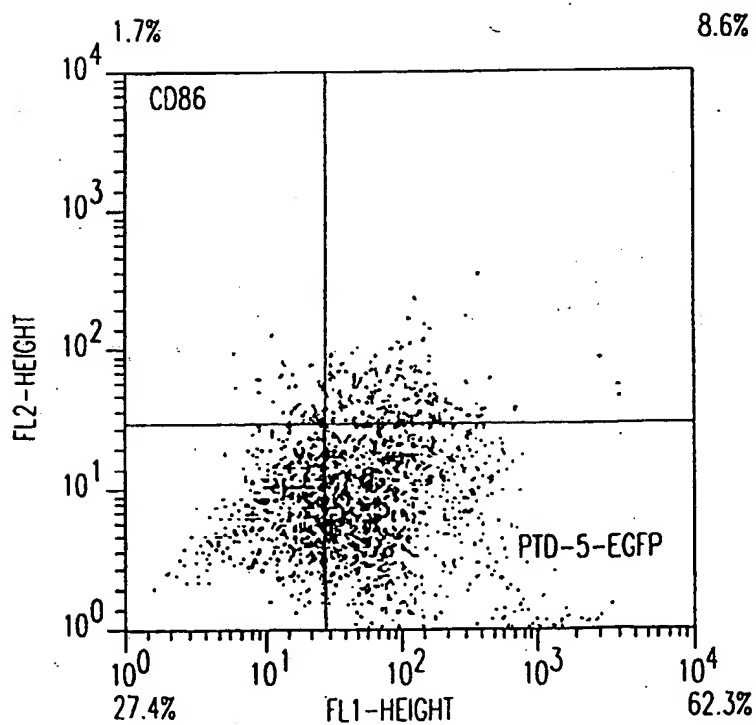


FIG. 22E

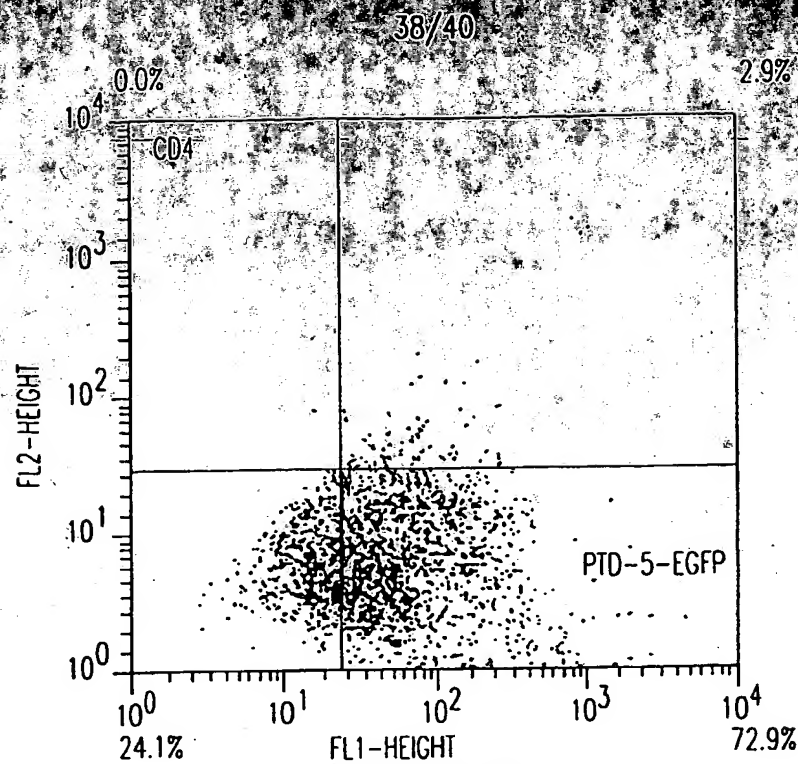


FIG. 22F

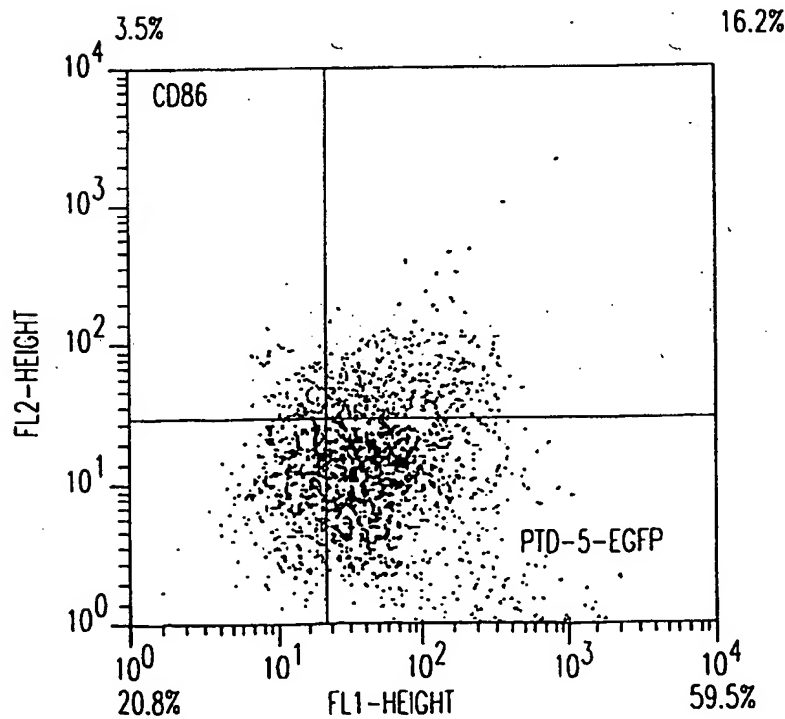


FIG. 22G

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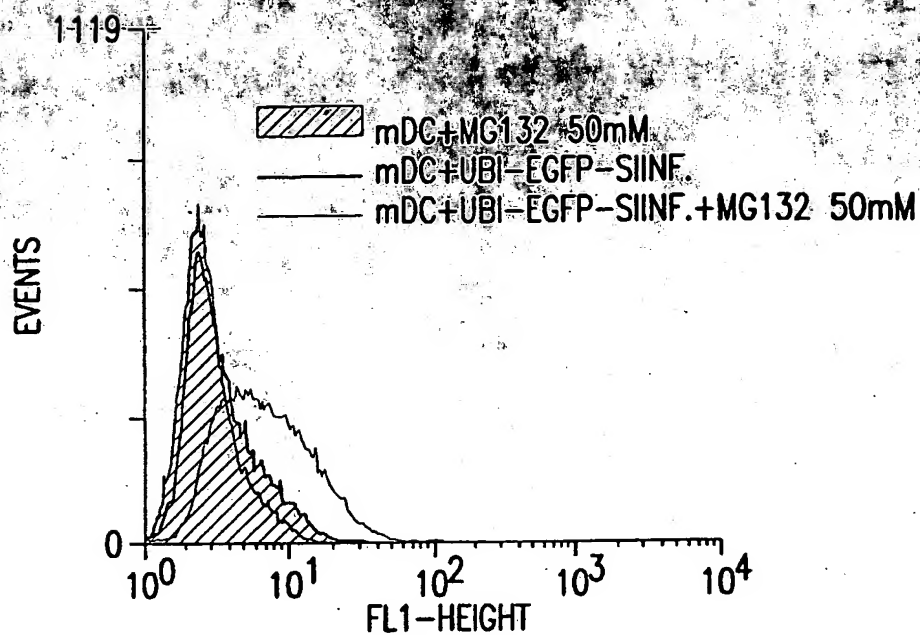


FIG. 23A

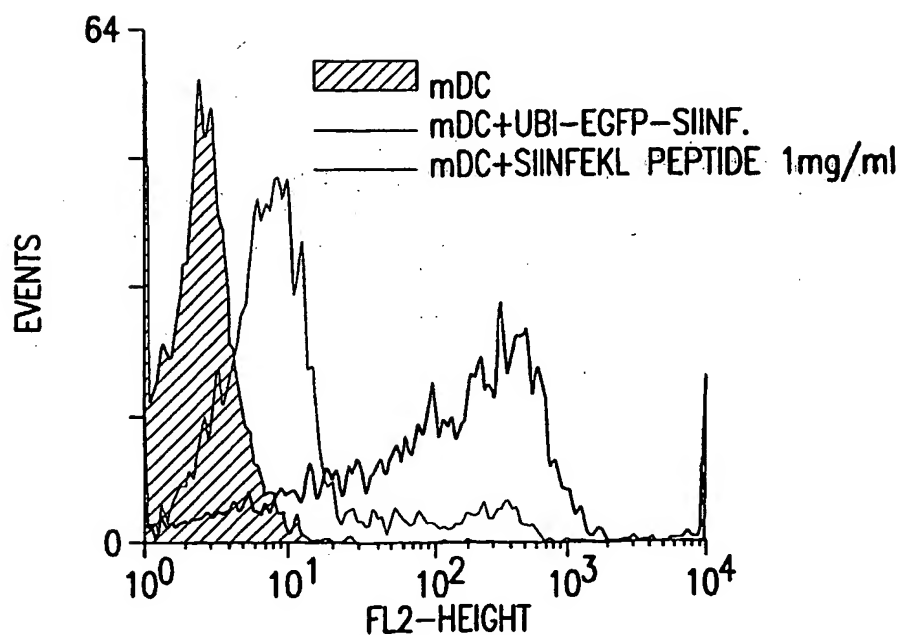


FIG. 23B

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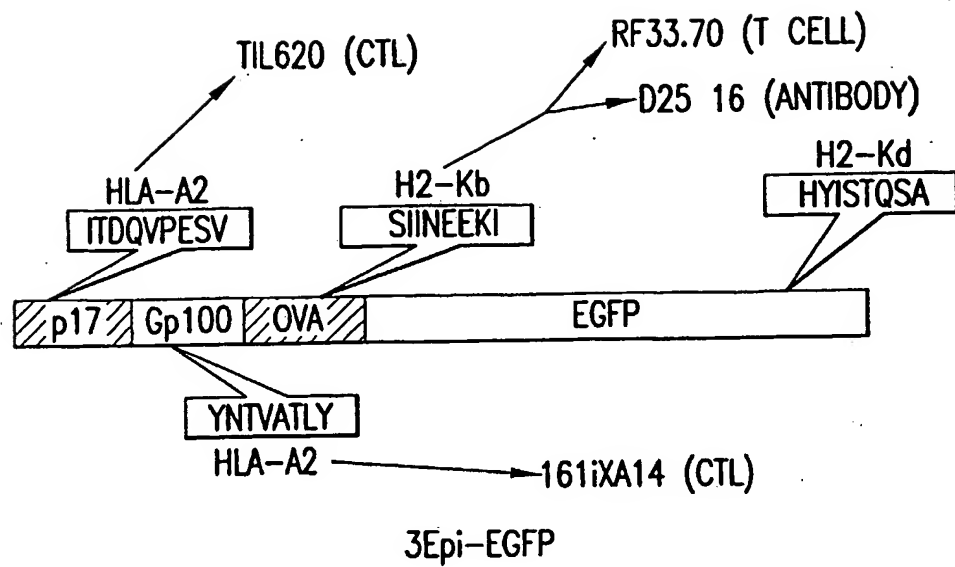


FIG.24

SEQUENCE LISTING

<110> Paul D. Robbins
Zhibao Mi
Raymond Frizzell
Joseph C. Glorioso
Andrea Gambotto

<120> IDENTIFICATION OF PEPTIDES THAT FACILITATE
UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT
OF PROTEINS, DNA AND VIRUSES

<130> 32573-A-A-PCT

<150> 60/151,980

<151> 1999-09-01

<150> 60/188,944

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International Bureau



(43) International Publication Date
8 March 2001 (08.03.2001)

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(10) International Publication Number
WO 01/15511 A3

- (51) International Patent Classification⁷: C07K 7/08. (74) Agents: SEIDE, Rochelle, K. et al.: Baker Botts LLP, 30 Rockefeller Plaza, New York, NY 10112-0228 (US).
C12N 15/10, A61K 47/48, C07K 14/47
- (21) International Application Number: PCT/US00/24034 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
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- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
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60/188,944 13 March 2000 (13.03.2000) US
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant: UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; 200 Gardner Steel Conference Center, Pittsburgh, PA 15260 (US).
- Published:
— with international search report
- (72) Inventors: ROBBINS, Paul, D.: 191 Main Entrance Drive, Mt. Lebanon, PA 15228 (US). MI, Zhibao; 221 Buchanan Place, Apt. C2, Pittsburgh, PA 15228 (US). FRIZZELL, Raymond; 5304 Forbes Avenue, Pittsburgh, PA 15217 (US). GLORIOSO, Joseph, C.: 202 Scarborough Drive, Cheswick, PA 15024 (US). GAMBOTTO, Andrea; 1250 South Negley Avenue, Pittsburgh, PA 15217 (US).
- (88) Date of publication of the international search report:
24 January 2002
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/15511 A3

(54) Title: IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

(57) Abstract: The present invention relates to internalizing peptides which facilitate the uptake and transport of cargo into the cytoplasm and nuclei of cells as well as methods for the identification of such peptides. The internalizing peptides of the present invention are selected for their ability to efficiently internalize cargo into a wide variety of cell types both *in vivo* and *in vitro*. The method for identification of the internalizing peptides of the present invention comprises incubating a target cell with a peptide display library, isolating peptides with internalization characteristics and determining the ability of said peptide to internalize cargo into a cell.

INTERNATIONAL SEARCH REPORT

International Application No

P. /US 00/24034

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K7/08 C12N15/10 A61K47/48 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 10485 A (SELECTIVE GENETICS INC) 4 March 1999 (1999-03-04) claims; example 12 ---	
A	IVANENKOV VASILY V ET AL: "Targeted delivery of multivalent phage display vectors into mammalian cells." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1448, no. 3, 11 January 1999 (1999-01-11), pages 463-472, XP002157575 ISSN: 0006-3002 abstract ---	
A	EP 0 903 408 A (BIOGEN INC) 24 March 1999 (1999-03-24) abstract ---	
	--- -/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

17 January 2001

Date of mailing of the international search report

25. 04 2001

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No

F /US 00/24034

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 51825 A (ELAN CORP PLC ;SEVESO MICHELA (IE); MAHONY DANIEL JOSEPH O (IE); A) 19 November 1998 (1998-11-19) abstract ---	
A	WO 98 51325 A (MAHONY DANIEL J O ;PATTERSON CATHERINE A (IE); ELAN CORP PLC (IE);) 19 November 1998 (1998-11-19) ---	
A	WO 99 06542 A (ALLEN JANET M ;LAVERTY EDWARD (GB); UNIV COURT OF THE UNIVERSITY O) 11 February 1999 (1999-02-11) -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/24034

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see further information sheet invention group 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 3-6,14-17,21,44,52 (all complete); 1,2,7-13,18-20, 22-43,45-51,54-59 (all partially)

INVENTION 1:

Peptidic compounds corresponding to SEQ IDs 1-5, facilitating the cellular internalization of cargo compounds linked thereto, methods for their identification by peptide display library, peptide-cargo complexes, DNA encoding for them and their use as pharmaceutical agents or as immunogens.

For the sake of conciseness, only the first subject is explicitly defined, the other groups being defined by analogy.

2. Claims: 53 (complete); 1,2,7-13,18-20,22-43,45-51, 54-59 (all partially)

INVENTION 2 to INVENTION 65:

Same as subject 1, but limited to SEQ IDs 6-75 in agreement with the list given in claim 1 of the application.

(e.i.: invention 2 is limited to peptides having SEQ ID 6, invention 3 to SEQ ID 7...invention 14 to SEQ ID 18, invention 15 to SEQ ID 25...invention 65 to SEQ ID 75).

Claim 53 refers specifically to SEQ ID 73 and will be searched as part of subject-matter of invention 63.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9910485	A	04-03-1999	AU 9039898 A EP 1009819 A NO 20000992 A	16-03-1999 21-06-2000 27-03-2000
EP 0903408	A	24-03-1999	AT 173016 T AU 667244 B AU 5083293 A CA 2135642 A DE 69321962 D DE 69321962 T DE 656950 T DK 656950 T EP 0656950 A ES 2123062 T FI 945248 A JP 2869396 B JP 10033186 A JP 2702285 B JP 7503617 T KR 153027 B NO 944273 A NZ 255831 A WO 9404686 A US 5674980 A US 5670617 A US 5652122 A US 5747641 A	15-11-1998 14-03-1996 15-03-1994 03-03-1994 10-12-1998 01-07-1999 14-03-1996 19-07-1999 14-06-1995 01-01-1999 05-01-1995 10-03-1999 10-02-1998 21-01-1998 20-04-1995 15-10-1998 17-02-1995 24-04-1997 03-03-1994 07-10-1997 23-09-1997 29-07-1997 05-05-1998
WO 9851825	A	19-11-1998	AU 7575698 A EP 0981649 A	08-12-1998 01-03-2000
WO 9851325	A	19-11-1998	AU 7494398 A EP 1019071 A	08-12-1998 19-07-2000
WO 9906542	A	11-02-1999	AU 8550398 A EP 1000142 A	22-02-1999 17-05-2000